

Functional effects of anti-neuronal antibodies in patients with encephalitis lethargica and related disorders associated with streptococcal infection

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Functional effects of anti-neuronal antibodies in
patients with encephalitis lethargica and related
disorders associated with streptococcal infection

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Queen Mary University of London

Submitted for the Degree of Doctor of Philosophy (PhD)

DEDICATION

I would like to dedicate this thesis to my MOTHER

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It would not have been possible to write this doctoral thesis without the help and support of people around me, to only some of whom it is possible to give particular mention here.

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ABSTRACT

Encephalitis lethargica affected a large number of people in the pandemic in the early 1900s (von Economo, 1930). Histological and biochemical data suggest that autoimmune mechanisms play an important role in this disorder and recently serum anti-basal ganglia antibodies (ABGA) have been detected in affected sporadic cases associated with evidence of recent streptococcal infection (Dale et al., 2004a). ABGA are also associated with other neuropsychiatric disorders including Sydenhams chorea, paediatric autoimmune neuropsychiatric disorders associated with streptococcal infections, Tourettes syndrome and obsessive compulsive disorder. The precise frequency, presentation, disease course, treatment response and causes of these disorders are still unknown. As ABGA are strongly associated with recent streptococcal infection, these disorders represent a potentially good model for the study of molecular mimicry and autoimmunity.

The present study focussed on various aspects of this group of disorders. Profiling of group A streptococcus isolates from both patients with postulated post-streptococcal disorders of the CNS and controls was done which highlighted differences in virulence factors like M protein and superantigens between the two groups. Also in the present study we demonstrated the pathogenicity of anti-neuronal antibodies found in patients in both an *in vivo* and *in vitro* setting. An animal model of the disorders was produced by passive transfer of antibodies from patients which resulted in symptoms reminiscent of diseases like encephalitis lethargica and dystonia. An active immunization animal model using GABHS proteins and recombinant proteins (putative autoantigens) was also developed. Furthermore, the autoantibodies from patients and animal models were analysed on both neuronal and non-neuronal cells where they demonstrated to have a functional effect on cytotoxicity, apoptosis, calcium flux and enolase activity. N-methyl D-aspartate glutamate receptor and voltage gated potassium channel have been recently been implicated in a

range of neurological disorders, hence we also tested the patient sera for antibodies against these receptors and found a group of patients to be positive.

In summary, EL and other ABGA-associated disorders are still an emerging entity, with major implications for neuropsychiatry. As auto-antibody mediated diseases respond to immunomodulatory therapy, identifying and defining the pathogenesis of these disorders is important so that patients can be appropriately treated.

ABBREVIATIONS

Acute rheumatic fever	ARF
Adenosine di-phosphate	ADP
Aldolase	Ald
Alzheimer's disease	AD
Amyotrophic lateral sclerosis	ALS
Antibody	Ab
Anti-basal ganglia antibodies	ABGA
Antigen	Ag
Anti-streptolysin O titre	ASOT
β -nicotinamide	β -NADH
Basal Ganglia	BG
Bovine serum albumin	BSA
Dithiotheitol	DTT
Distilled water	dH ₂ O
Calcium	Ca ²⁺
Calcium/Calmodulin-dependent protein kinase II	CaMKII
Encephalitis lethargica	EL
Endoplasmic reticulum	ER
Enolase	Eno
Fluorescein isothiocyanate	FITC
Gamma-Amino-Butyric Acid	GABA
Group A beta haemolytic streptococcus	GABHS
Haematoxylin and Eosin	H&E
Hours	h
Huntington's disease	HD

Hydrochloric acid	HCl
Immunocytochemistry	ICC
Immunoglobulin G	IgG
Lactate dehydrogenase	LDH
Kilodalton	kDa
Minutes	Min
Molar	M
Multiple sclerosis	MS
Neurofilament	NFL
Obsessive compulsive disorder	OCD
Optical Density	OD
Paediatric Autoimmune Neuropsychiatric Disorders Associated with Streptococcal Infections	PANDAS
Parkinson's disease	PD
Paraformaldehyde	PFA
Potassium chloride	KCl
Pyruvate kinase	PK
Revolutions per minute	RPM
Room temperature	RT
Seconds	s
Systemic lupus erythematosus	SLE
Superantigens	SAg
Sydenham's chorea	SC
Tetramethylrhodamine-5-(and 6)-isothiocyanate	TRITC
Tourette's syndrome	TS

CHAPTER 1

INTRODUCTION

1.1 THE BASAL GANGLIA

1.1.1 Background

The basal ganglia are large subcortical gray matter masses in the brain comprising of the caudate nucleus, the putamen, the globus pallidus and the nucleus accumbens (Ringwood and Serra-Mestres, 2002). The basal ganglia have been considered an important area in motor control of musculature since the 19th century (Ferrier et al., 1876). They are also involved in psychomotor behaviour including motor learning, sequencing and movements, attention allocation and filtering, working memory and implicit learning and memory (Alexander et al., 1986; Brown et al., 1999; Nakano, 2000; Ringwood and Serra-Mestres, 2002). The basal ganglia do not have direct output connections to the spinal cord so the motor functions are mediated through the pyramidal system of neurones (Percheron et al., 1994).

The neurones comprising the basal ganglia are arranged in a complex system but the efferent neurones are GABAergic medium spiny neurones (Parent et al., 1993). These neurones contain the inhibitory neurotransmitter gamma-amino-butyric acid (GABA) and can be typed depending on the dopamine receptor type they contain (Albin et al., 1989; Ribak et al., 1979). Neuronal function within the basal ganglia is modulated by dopaminergic innervations from both within and outside the striatum, especially from the substantia nigra (Cossette et al., 1999). The main transmission system of the basal ganglia originates in the neocortex and the basal ganglia receive inputs from the associative areas of the neocortex and sensori-motor cortex (Gerfen, 1984; Haber et al., 1995). The cortex

utilises glutamate as the neurotransmitter and interact with the medium spiny neurones in the striatum (Bouyer et al., 1984; Cherubini et al., 1998).

1.1.2 Basal Ganglia Disorders

The consequences of disorders affecting the basal ganglia are not completely understood but include hypo and hyperkinetic movement abnormalities and a range of neuropsychiatric symptoms (Dale and Brilot, 2012). The neurotoxin, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) has given some insights into the relationship of the basal ganglia to Parkinsons disease (PD), a classical hypokinetic movement disorder characterised by rigidity and slow movements. MPTP causes extensive neuronal dopaminergic degeneration and a Parkinsonian phenotype due to over-reactivity of the neurones in the striatum and dysregulation of dopaminergic neurones (Dale and Brilot, 2012, Herrero et al., 1993).

Overt damage to the basal ganglia resulting in tissue lesions results from infections, tumours, vascular disorders, genetic disorders and trauma. Such lesions commonly result in apathy and loss of self-initiative (abulia) (Bhatia et al., 2004). The movement disorder dystonia, an extrapyramidal movement disorder with slow and sustained muscle contractions, is also common (Bhatia et al., 1994). The other common outcomes of basal ganglia lesions are chorea (fast, purposeless movements-hyperkinetic disorder) and athetoid-type movement disorder (slow, sinuous movements), (Destée et al., 1990; Denny-Brown., 1968). Disease of the basal ganglia associated with progressive neuronal loss and a genetic pathology or association, include Huntingtons disease (HD). This is typically a progressive hyperkinetic movement disorder with associated cognitive loss and dementia (Graveland et al., 1985). Wilson's disease is another genetic disorder associated with

abnormal copper metabolism and results in degeneration of the putamen and globus pallidus causing tremor and rigidity (Wilson, 1912; Giagheddu et al., 2001).

Psychiatric symptoms such as obsessive-compulsive disorder (OCD) and behavioural abnormalities have also been described associated with basal ganglia lesions (Laplane et al., 1989). The reason for the psychiatric abnormalities is that basal ganglia circuits have functional roles in oculomotor, pre-frontal, and cingulate pathways which are central to attention, learning and behavioural control (Brown et al., 1999; Gerfen, 1984; Haber et al., 1995; Yamashiro et al., 1997). Disruption in these circuits can therefore result in a range of extrapyramidal movement disorders and neuropsychiatric symptoms of which Sydenham's chorea (SC) is a good example. This disorder has been proposed as having an immune mediated pathogenesis (Kiplan et al., 2003).

Movement disorders (such as Huntingtons and Parkinsons diseases) occur due to damage to the basal ganglia and therefore it is proposed that Tourettes syndrome (TS) is linked to the basal ganglia (Peterson et al., 2003). In support of this hypothesis, studies have shown tic like behaviours in humans and animals are associated with electrochemical manipulation and lesions of the basal ganglia. Furthermore, neuroleptic drugs which treat tic disorders, work by blocking dopamine receptors in the basal ganglia (Peterson et al., 2003). Despite this strong circumstantial evidence, investigations into basal ganglia abnormalities in TS patients have produced conflicting results. Some studies have shown that the caudate nucleus is significantly reduced in size across all age groups with TS and that the left lenticular nucleus, putamen and globus pallidus are significantly smaller in TS adults (Peterson et al., 1993; Peterson et al., 2003). Other studies disagree and claim basal ganglia volumes are not significantly different in TS patients compared to controls (Singer et al., 2005).

1.1.3 Anti-basal Ganglia Antibodies

Anti-basal ganglia antibodies (ABGA) are anti-neuronal autoantibodies directed against the basal ganglia. They have been associated with movement disorders and psychiatric disturbances in adults and children postulated to be occurring as a result of infection, particularly with group A beta-hemolytic streptococcus (GABHS). Autoimmune mechanism involving molecular mimicry has been proposed. These antibodies have been shown to bind to common neural autoantigens of molecular weight 40, 45, 60 and 98 kDa (Church et al., 2003). Studies have shown that ABGA are specific for several antigens in TS and obsessive compulsive disorder (OCD) patients, including putamen and proteins of molecular weight 60, 67 and 83 kDa (Singer et al., 1998; Church et al., 2003; Hoekstra et al., 2003). ABGA antigens have also been identified in SC, with molecular weights of 40, 45 and 60 kDa (Church et al., 2002). Subsequent studies have identified these as the glycolytic enzymes aldolase C, neuronal enolase and pyruvate kinase M1 respectively (Dale et al., 2004). In addition, ABGA have also been observed in patients with acute disseminated encephalomyelitis (ADEM), an autoimmune inflammatory disorder of the nervous system (Dale et al., 2001). This study demonstrated that ABGA are significantly elevated in ADEM patients compared to controls and that they bind to basal ganglia antigens with molecular weights of 60, 67 and 80 kDa (Dale et al., 2001). This study suggested that ADEM should be included within the post-streptococcal sequelae group and also supports other studies which propose that the 60kDa antigen could be a critical factor in anti-neuronal autoimmune responses (Singer et al., 1998; Singer et al., 1999; Trifiletti and Bandele, 2000; Church et al., 2003). As mentioned earlier, one study reported this to be pyruvate kinase M1, however a different study suggests it could also be human heat shock protein 60 (hsp60) (Hoekstra et al., 2003). This latter study observed higher anti-hsp60 titres in patients with tics but not with attention deficit hyperactivity disorder (ADHD) and OCD.

Hsp60 is ubiquitously present in tissue, where it functions as a molecular chaperone protein, involved in protein assembly and folding. It can be released extracellularly where it is thought to protect cells and prevent damage in response to toxic agents. Antibodies to hsp60 are extremely common and bacterial and parasitic hsp60 show a high degree of homology with human hsp60. Indeed several rheumatic autoimmune diseases are linked with abnormal reactivity to hsp60, including severe coronary heart diseases, juvenile chronic arthritis and carotid (Hoekstra et al., 2003).

The blood-brain barrier (BBB) results from the selectivity of the tight junctions between endothelial cells in CNS vessels that restricts the passage of solutes. Normally, the CNS would be inaccessible to leukocytes due to this barrier, however, in various diseases the barrier function is compromised examples of which include epilepsy, multiple sclerosis, meningitis, Alzheimer's disease and progressive multifocal leukoencephalopathy (PML) (Luissint et al., 2012). It has been suggested that rather than being a disease of the immune system these might be diseases of the BBB. In epilepsy studies have implicated the interactions between albumin, a common blood protein and astrocytes (Ivens et al., 2007). Similarly loss of function of vascular P-glycoprotein an ATP-binding cassette transporter which is highly expressed at the BBB has been reported during neuroinflammation in models of multiple sclerosis (Kooij et al., 2011). Notably, lymphocyte interaction via endothelial intracellular adhesion molecule -1 (ICAM-1) caused the activation of a nuclear factor kappa B (NF-kappaB) signalling pathway, which resulted in endothelial P-gp malfunction (Kooij et al., 2010).

1.2 AUTOIMMUNITY

1.2.1 What is Autoimmunity?

Autoimmunity is a condition triggered by the immune system initiating an attack on self-molecules due to deterioration of immunologic tolerance to auto-reactive immune cells (Atimal and Shoenfeld, 2004). Some examples of autoimmune diseases include Coeliac disease, rheumatoid arthritis (RA), diabetes mellitus type 1 (IDDM), systemic lupus erythematosus (SLE), Sjögrens syndrome, Churg-Strauss Syndrome, Graves disease, idiopathic thrombocytopenic purpura (PubMed Health) . Although autoimmune disorders can affect an individual of any gender, age or race certain people are more susceptible taking into account certain risk factors associated with these disorders (figure below). For example it is known that females, young to middle-aged individuals, individuals with a family history and individuals from African American/American Indian/ Latino ethnicity are at an increased risk of developing an autoimmune disorder (Page et al., 2012, Gleicher and Barad, 2007). Also exposure to certain medications (e.g. procainamide) or metals (e.g. mercury) and bacterial and viral infections in a genetically susceptible individual could contribute towards an increased risk (Bernstein, 1985).

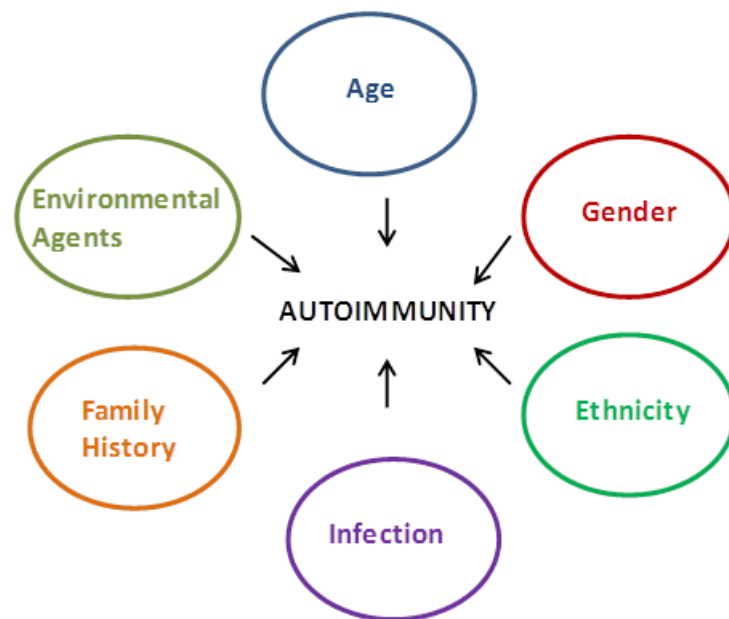


Figure 1.1: Risk factors associated with autoimmune disorders. The figure above depicts some of the risk factors found to be associated with the development of an autoimmune disorder.

The exact aetiology of autoimmune diseases is not known. However, various theories have been proposed that include sequestered antigen, escape of auto-reactive clones, loss of suppressor cells, cross reactive antigens including altered self antigens (chemical and viral infection) and exogenous antigens (pathogens) (Ghaffar and Ngarkatti, 2010, Gleicher and Barad, 2007).

Witebsky's postulates

Autoantibodies and to some extent self-reactive T cell clones have been detected in patients with a variety of diseases. However, a causative role in disease development has not always emerged and a set of criteria had to emerge to distinguish *bona fide* autoimmune diseases from other diseases in which some feature of autoimmunity is

present but in which a causative role is lacking, these are termed as Witebsky's postulates (Rose and Bona, 1993):

- Direct evidence from transfer of pathogenic antibody or pathogenic T cells.
- Indirect evidence based on reproduction of the autoimmune disease in experimental animals.
- Circumstantial evidence from clinical clues.

1.2.2 Bacteria and Autoimmunity

In addition to viruses, a number of other pathogens including bacteria have been associated with autoimmune diseases involving chronic inflammation and demyelination. Extensive research is being done to better understand the possible mechanisms of bacterial involvement as aetiological agents or in the exacerbation of these diseases. Variety of bacterial infections have been linked with rheumatoid conditions (Sherbet, 2009). Infection by *Mycoplasma pneumoniae*, *M. salivarium*, and *M. fermentans*, has been strongly associated with rheumatoid arthritis (Furr et al., 1994; Haier et al., 1999). *Proteus mirabilis* has also been implicated in the pathogenesis of RA and in osteoarthritis (OA) (Tiwana et al., 1997; Wanchu et al., 1997). *Borrelia burgdorferi*, which is transmitted by ticks, typically causes cutaneous disease, encephalitis and arthritis (Steere, 2001). *Borrelia burgdorferi sensu stricto* infection is usually associated with a reactive arthritis, but 10% of these patients also develop an autoimmune form of Lyme disease arthritis. This form of arthritis is associated with constant inflammation and is treatment resistant (Guerau-de-Arellano et al., 2002; Steere, 2001).

Bacterial infections have been found to be associated with autoimmune conditions besides RA. Certain members of the *Enterobacteriaceae* family are linked with Kawasaki syndrome

and Graves' disease (Sherbet, 2009). *Haemophilus influenzae*, *Campylobacter jejuni*, and *M. pneumoniae* have been highlighted as the possible causative agents of a demyelinating neuropathy called Guillain-Barre (GB) syndrome (Yuki, 2007). Recently studies involving serology and PCR have provided evidence of *Chlamydomphila pneumoniae*, *Mycoplasma* species, *Borrelia burgdorferii* and human herpesvirus-1 and -6 in multiple sclerosis (MS), amyotrophic lateral sclerosis (ALS) Alzheimers and Parkinsons disease (Nicolson, 2008; Sherbet, 2009).

Group A beta-haemolytic streptococcus (GABHS) or *Streptococcus pyogenes* has been linked with autoimmune disease in humans with particular involvement of the heart, joints, and brain.

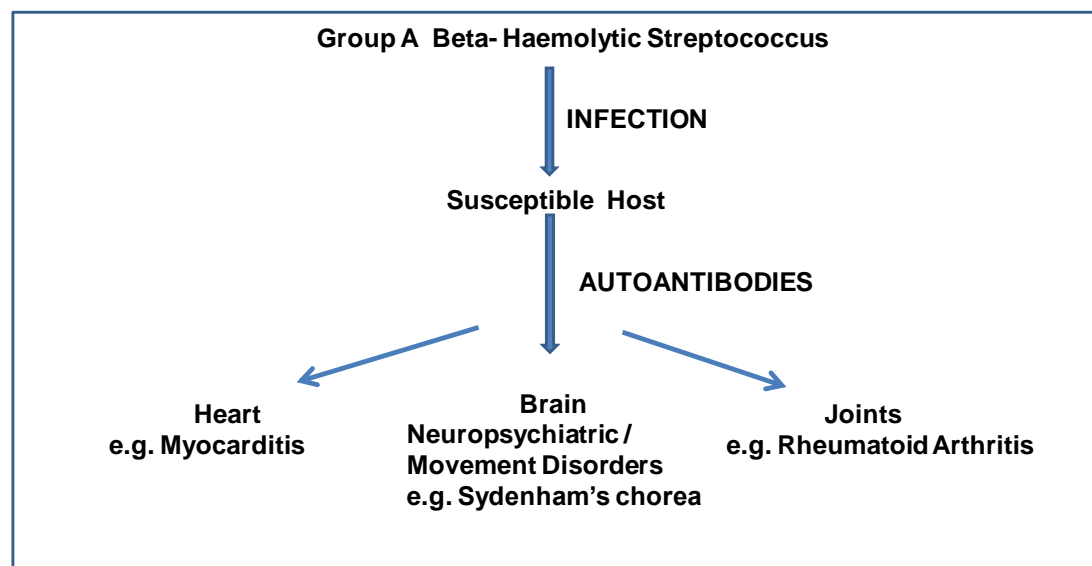


Figure 1.2: Postulated autoimmune aetiology of post-streptococcal disorders. GABHS infection in a susceptible host leads to molecular mimicry thereby raising autoantibodies leading to disorders such a rheumatic fever characterized by carditis, neuropsychiatric disorders like Sydenhams chorea (SC) and rheumatoid arthritis.

1.3 GROUP A BETA-HAEMOLYTIC STREPTOCOCCUS (GABHS)

1.3.1 Background

Streptococcus is a genus of spherical Gram-positive bacteria. The bacteria is non-motile and non-sporeforming and characteristically forms pairs or chains during growth (Todar, 2008). Streptococci are oxidase and catalase negative and many are facultative anaerobes. They are generally strong fermenters of carbohydrates resulting in the production of lactic acid, a property employed in the dairy industry (Greenwood et al., 2007). Some streptococci are important ecologically as part of the normal microbial flora of animals and humans, while others are associated with important human diseases ranging from subacute to acute or even chronic, attributable in part to infection by the bacteria and in part to sensitization to them (Brooks et al., 2007).

Lancefield group A streptococci consists of a single species, *Streptococcus pyogenes* which typically produces a large zone of beta-haemolysis when cultured on blood agar plates, and is therefore also called Group A beta-haemolytic Streptococcus (GABHS) (Ryan and Ray, 2004). It is associated with a wide range of suppurative infections in the skin and respiratory tract, life threatening soft tissue infections and certain types of toxin-associated reactions (Hayes et al., 2001). Acute *S. pyogenes* infections may take the form of pharyngitis, impetigo, cellulitis or scarlet fever (Cunningham, 2008). Invasive infections can result in streptococcal toxic shock syndrome, necrotizing fasciitis or myositis (Hahn et al., 2005).

GABHS express a number of cell surface components and extracellular products that play an important role in its pathogenesis and in the immune response of the host as discussed previously. Although the pathophysiology of post-streptococcal disorders is not completely

understood, antigenic mimicry is proposed to be the triggering cause of autoimmunity (Hahn et al., 2005; Greenwood et al., 2007).

Evidence suggests that GABHS contain antigens which are similar enough to host antigens and can stimulate B and T cells to respond to self. The streptococcal M protein, N-acetylglucosamine, and group-specific carbohydrate contain antigenic epitopes that mimic those of mammalian muscle and connective tissue. Of all the streptococcal antigens involved in molecular mimicry, the M protein has been best investigated and characterized (Bordeur, 2003).

1.3.2 Virulence factors

GABHS elaborates a number of cell surface components and extracellular products that play a central role in the pathogenesis of infection and the immune response of the host (Stevens et al., 2012). Although some factors are expressed by all clinical isolates others are variably present among *S. pyogenes* strains. This variation is owed to the horizontal transfer of virulence genes among the strains primarily by transduction. This explains the temporal variations in the prevalence of severe infections and sequelae together with differences in the virulence of individual strains (Lamagni et al., 2008).

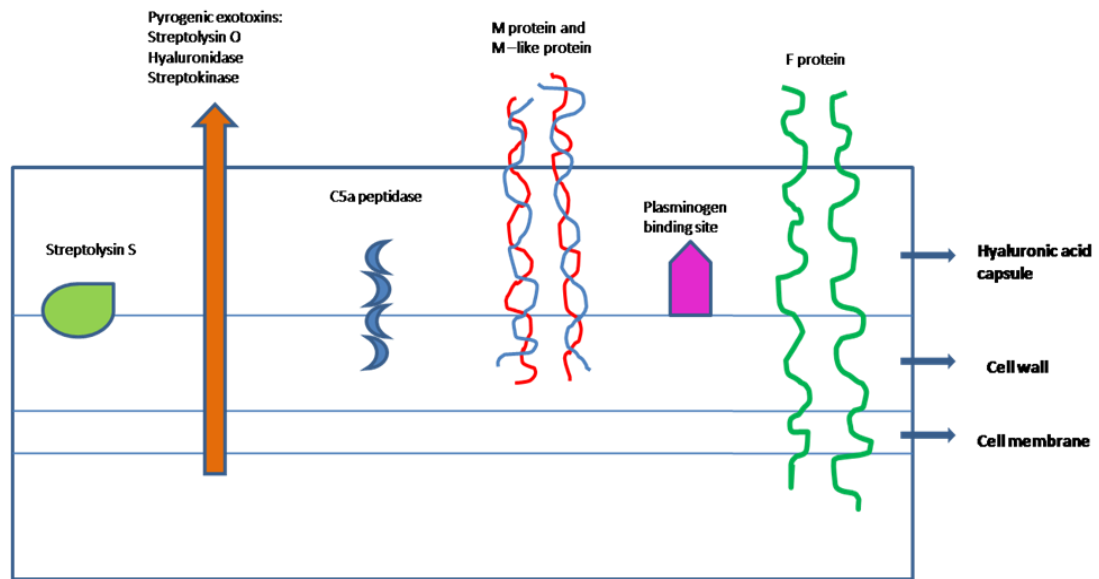


Figure 1.3: GABHS virulence factors. Schematic diagram showing some of the virulence factors associated with GABHS.

1.3.2.1 Streptolysins

GABHS expresses two distinct streptolysins, Streptolysin O and S both of which lyse erythrocytes, polymorphonuclear leucocytes and platelets by forming pores in their membrane (Bisno et al., 2003).

Streptolysin O (SLO)

Streptolysin O (oxygen labile) is a protein that is haemolytically active in the reduced state (available –SH groups) but rapidly inactivated in the presence of oxygen (Thelestam and Molby, 1980). Cytolysin-mediated translocation (CMT) of GABHS uses streptolysin O to translocate the bacterium's nicotinamide adenine dinucleotide-glycohydrolase (SPN) into the host cell cytosol, resulting in cell death (Magassa et al., 2010). Analysis of GABHS virulence factor mutants, heterologous expression, and purified toxin studies identified the pore-forming streptolysin O both necessary and sufficient for the apoptosis-inducing phenotype (Timmer et al., 2009). Streptolysin O is also capable of affecting the immune

response by impairing neutrophil function and inducing monocytes to produce tumour necrosis factor (TNF) and keratinocytes to produce a host of inflammatory cytokines (Bremm et al., 1987; Hackett et al., 1993; Ruiz et al., 1998).

Streptolysin S (SLS)

The molecular identity of Streptolysin S was revealed only recently and characterization of partially purified preparation has suggested it to be 2.8 kDa protein (Betschell et al., 1998; Nizer et al., 2000; Wessels MR, 2005). SLS is known to be responsible for the beta-haemolysis around streptococcal colonies on blood agar plates and plays a role in inhibition of neutrophil recruitment during the early stages of infection (Lin et al., 2009). It is known to be produced by a vast majority of GABHS strains however, SLS negative strains (non-haemolytic) have been more associated with human infections which suggests that SLS might not be essential for virulence (Sierig et al., 2003; Wessels et al., 2005).

1.3.2.2 C5a Peptidase

Virulent strains of *S.pyogenes* are found to produce a C5a peptidase (SCPA) which is bound to their surface and specifically cleaves human C5a one of the principle chemo-attractants of the phagocytic cell (Brown et al., 2005). Activation of the complement system by either the classical or alternative pathway generates C5 convertase that proteolytically liberates C5a, a 74-residue chemotactic peptide (O'Connor and Cleary, 1987). A chemical gradient of C5a attracts phagocytic polymorphonuclear leukocytes to the site of infection to form the first line of defence against persistent colonization and invasion of deeper tissues (Cleary et al., 1992).

1.3.2.3 Hyaluronic Acid Capsule and Hyaluronidase

Some strains of GABHS form a capsule made up of hyaluronic acid (Sugahara et al., 1979; Schager et al., 1996). The capsule presenting strains grow as mucoid colonies on blood agar plates and the capsule of GABHS has been shown to enhance virulence in systemic and intranasal challenge models (Sugahara et al., 1979; Schager et al., 1996). The encapsulated strain has been shown to be highly virulent, producing extensive local tissue necrosis and secondary bacteraemia, compared to the un-encapsulated strain that produced fewer and less severe local lesions with no animals developing bacteraemia (Blank et al., 2005). The capsule is identical to the hyaluronic acid of the host connective tissue and is not immunogenic. In this way the bacteria disguise themselves with an immunological self substance (Schrager et al., 1996). Secreted hyaluronidase is released by the bacterium to degrade hyaluronic acid the ground substance of host connective tissue. During infections of the skin, serum antibody titres to hyaluronidase show a significant rise (Harris et al., 1950).

1.3.2.4 F Protein

F protein has been shown to be surface-expressed and to bind tightly to fibronectin, the extracellular matrix protein to which GABHS bind (Hanski et al., 1992). M protein is not expressed under conditions that promote expression of F protein (high O₂ and low CO₂), and vice versa. The interaction between the streptococcal F protein and host cell fibronectin also mediates internalization of the bacteria into the host cells (Stevens et al., 2000; Greenwood et al., 2007).

1.3.2.5 Streptokinase (Fibrinolysin)

Streptokinase is produced by many strains of GABHS. It transforms the plasminogen of human plasma into plasmin an active proteolytic enzyme that digests fibrin and other proteins (Tewedros et al., 1995). GABHS uses host plasmin to hinder the build-up of fibrin barriers resulting in soft tissue infections that are more diffused and often spread readily than well localized abscesses (Bardia, 2007).

1.3.2.6 Superantigens (SAg)

Microbial superantigens (SAg) are a family of protein exotoxins that cause non-specific activation of T-cells resulting in polyclonal T cell activation and massive cytokine release (Llewelyn and Cohen, 2002). These extracellular pyogenic exotoxins are important as they cause direct tissue damage by initiation of apoptotic pathways or disruption of mammalian cell-walls (Hallas, 1985; Saouda et al., 2001). These immunostimulatory molecules activate large numbers of T cells by binding simultaneously to both major histocompatibility (MHC) class II molecules and T cell receptors (TCR) (Brouillard et al., 2007). However, binding of superantigens to MHC class II molecules requires no prior processing and occurs outside the antigen-binding groove (Pullen et al., 1990). Compared to a normal antigen-induced T-cell response where 0.001-0.0001% of the T-cells are activated, SAg are capable of activating up to 20% of the T-cells (Li et al., 1998). Conventional antigens, when presented to the major histocompatibility complex (MHC) on antigen-presenting cells, require recognition by all five variable elements ($V\beta$, $D\beta$, $J\beta$, $V\alpha$, $J\alpha$) of the TCR in contrast to SAg which are primarily recognized by only the $V\beta$ chain of the TCR (Davis and Bjorkman, 1988).

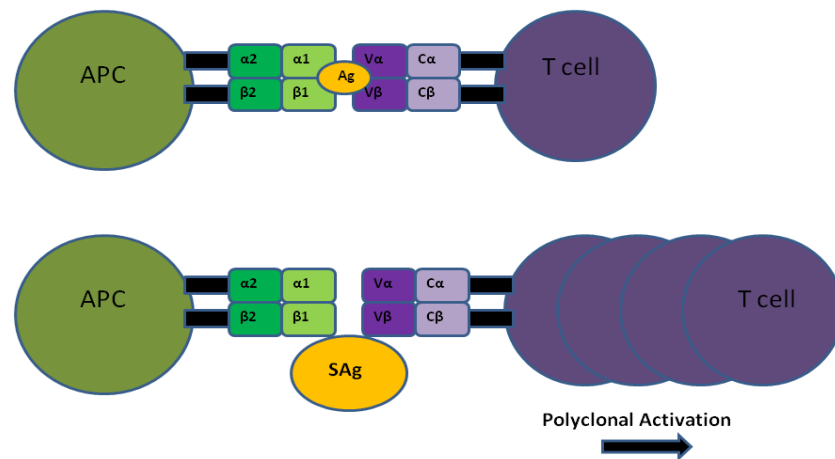


Figure 1.4: Bioactivity of Superantigens (SAG). Diagrammatic representation of non-specific activation of T-cells caused by streptococcal SAg. Once secreted SAg require no prior processing to interact with the antigen-presenting cell (APC). The activity of SAg depends on its ability to bind both the MHC class II and TCR outside the antigen binding groove.

To date eleven distinct superantigens have been identified to be produced by GABHS including the pyrogenic exotoxins (Spe) A, C, G, H, I, J, K, L, M, ssa and smez (Norrby-Teglund et al., 2001). The distribution of these superantigens varies between different streptococcal strains except exoproteins SpeB and SpeF which are chromosomally encoded and function as a cysteine protease and DNase, respectively, however neither of the two is a true SAg (Sriskandan et al., 2007).

Microbial superantigens are medium size proteins (molecular weight 22-29 kDa) characterized by high resistance to proteases and to denaturation by heat (Papageorgiou and Acharya, 2000). Comparison of those structures already solved indicates conserved two-domain architecture (N terminal β -barrel domain and C-terminal β -grasp domain) and a long, solvent-accessible α -helix spanning the centre of the molecule (Baker et al., 2004, Bulliard et al., 1997). A common feature of several superantigens is the presence of a highly flexible disulphide loop within the N-terminal (Reda et al., 1994). Interestingly, this loop has

been implicated in the emetic properties of these bacterial toxins (Reda et al., 1994). Key regions interact with host major histocompatibility complex (MHC) class II molecules and the variable region of the T cell receptor (TCR) beta (TCRV β) chain, thereby bringing MHC class II molecule and TCR together (Sriskandan et al., 2007). Despite this large amount of crystallographic information, many questions regarding the exact mode of superantigen function remain unanswered (Solanki et al., 2008).

Binding affinity of superantigens for human leucocyte antigen (HLA) class II is believed to underlie the magnitude of superantigen induced T cell activation (Michaelson et al., 2011). Bacterial superantigens are known to exhibit a clear binding preference for human MHC class II molecules compared to murine (Faulkner et al., 2005). The difference between mice and human response to SAg is most likely related to the difference in the MHC class II structures (Sriskandan et al., 2001). Mice expressing HLA class II transgenes (C57BL/10.DQ8) exhibit greater sensitivity to superantigen SPEA both *in vitro* and *in vivo* as compared to wild-type mice (Sriskandan et al., 2001). Some earlier studies have also shown that immunization of mice with bacterial SAg lead to polyclonal expansion of specific V β T cell subsets approximately 2 days later followed by clonal deletion and a period of superantigen specific T cell anergy that eventually recovers (MacDonald et al., 1991). Despite these findings it is difficult to extrapolate these immunological observations to the clinical setting as mice are comparatively resistant to superantigens and immunization with SAg cannot mimic the continuous release of the toxin into the tissue that occurs during an infection (Vabulas et al., 1996).

Sub-lethal doses of superantigen and lipopolysaccharide (LPS) can induce a synergistic shock in rodents if co-administered (Bohach et al., 1990). The mechanism underlying this phenomenon is not completely understood. A recent study using human monocytes pre-

exposed to superantigens SpeA, SmeZ, SEA, SEB for 3 hours showed a significantly exaggerated TNF- α response following LPS exposure (Hopkins et al., 2005). It is hypothesised that the synergy between LPS and superantigens is due to enhanced pattern recognition of LPS as superantigens presenting via MHC class II complex which leads to increased expression of toll like receptor 4 (TLR4) the pattern recognition receptor of LPS (Hopkins et al., 2005). In the HLA-DQ8 transgenic model described above (Sriskandan et al., 2001) the mice pre-exposed to D-galactosamine (D-gal) demonstrated an enhanced sensitivity to SPEA. D-gal is used to sensitize mice to acute lethal effects of LPS and more recently to the effects of injected staphylococcal superantigens (Miethke et al., 1992).

Further research into the structure-function relationships of bacterial superantigens is vital for a number of reasons. Firstly to identify potential new treatments for the growing list of diseases in which these toxins are implicated. Superantigens can down regulate the immune response following acute exposure resulting in anergy and /or deletion of T cells, this potential of SAg can be exploited to eliminate specific populations of T cells involved in autoimmune disorders (Sriskandan et al., 2001; Michaelson et al., 2011). SAg or superantigen toxoids might also be suitable vaccine targets (Sriskandan et al., 2007). Delivered in such a way as to trigger only MHC class II antigen signalling, SAg may prove to be excellent vaccine adjuvants or stimulants (Hopkins et al., 2005).

1.3.2.7 Emm gene and M protein

Emm gene

The *emm* gene is located on the chromosome near the GABHS multiple gene regular and encodes the M protein. A single strain may express up to three distinct *emm* genes with skin and throat serotypes distinguished by genetic markers located in the 3' end. This portion

encodes the cell-associated regions of the M protein and is highly conserved between different serotypes. The nucleotide sequences of the 3' ends are divided into 4 phylogenetic lineages, each representing a subfamily of *emm* genes (SF1 –SF4). The content and relative chromosomal arrangement of the SF genes exists in 5 patterns (A-E) with patterns A, D, and E the most common. Strains with patterns A-C are most likely to be isolated from the throat, whereas D pattern strains are associated with impetigo. Strains with pattern E belong in a third group of GAS types but it is unclear which tissue sites these streptococci prefer (Bessen and Lombroso, 2001).

An international protocol for amplifying and sequencing the *emm* gene has been developed at the centre for disease control and prevention (CDC). Sequences can be compared against those in the CDC *emm* sequence database using their basic local search alignment tool (BLAST). This database only includes sequences that have been obtained by the CDC streptococcal genetics laboratory. BLAST searches can be performed at <http://www.cdc.gov/ncidod/biotech/strep/strepblast.htm>. An *emm* sequence is defined as a particular serotype if it shares 95% identity with a database sequence over the first 160 bases, or as many bases close to this figure.

M Protein

Streptococcal M protein is a major surface protein and virulence factor of group A, C and G β -haemolytic streptococci (BHS) (Cunningham MW, 2000; Anand et al., 2012). It plays a central role in the pathogenesis by virtue of its anti-phagocytic properties. The M protein is attached to the cell membrane via an LPSTGE motif and consists of 2 polypeptide chains arranged in a α -helical coiled coil configuration (Anand et al., 2012). These polypeptide chains pass through the cell wall and appear as fibrils on the cell surface (Cunningham MW, 2000; Bisno et al., 2003). The primary structure of the M protein comprises 4 repeat

regions (A-D) which differ in amino acid sequence and size. Within these repeat blocks are 7 residual repeats of non-polar amino acids. The carboxy-terminal region corresponds to the portion located within the cell wall and cell membrane, while the amino-terminal region extends from the cell wall into the environment (Cunningham, 2000; Bisno et al., 2003). The carboxy-terminal portion is highly conserved between group A, C and G BHS, whereas the hypervariable region in the amino-terminal portion varies significantly between isolates. This hypervariable region corresponds to repeat region A (also known as the N terminal) and is used to serotype group A, C and G BHS (Cunningham, 2000; Bisno et al., 2003). Approximately a 124 types of M proteins have been discerned by serological means and gene sequencing, which could lead to recurrent infection in the individual with strains expressing different versions of this protein (Bisno et al., 2003; Ryan, 2004).

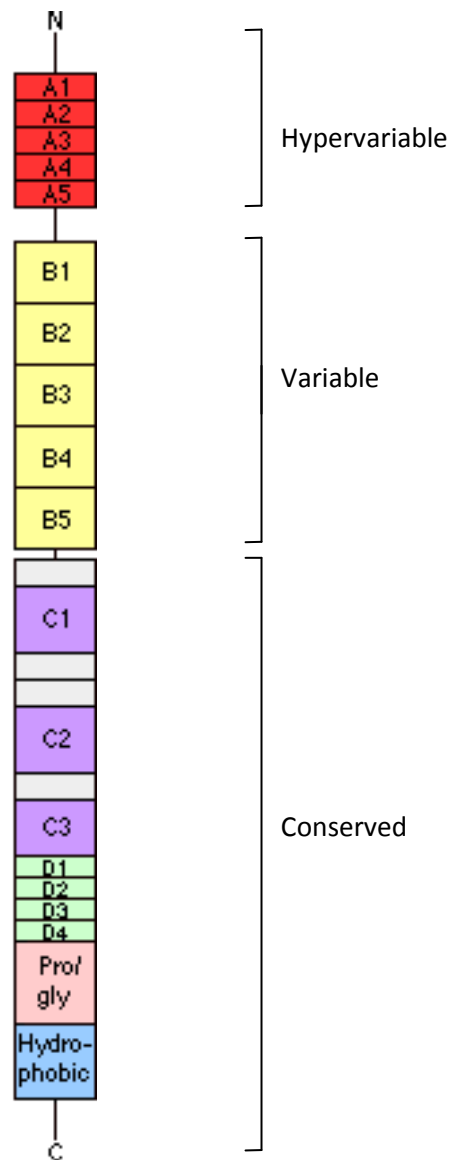


Figure 1.5: Structure of a streptococcal M protein. A typical streptococcal M protein showing the repeat regions (A-D), the proline and glycine rich peptidoglycan region and a hydrophobic portion.

The primary function of the M protein is to inhibit phagocytosis, which enables GABHS to survive in host infected tissues (Zartash Zafar Khan, 2009). Some strains produce two different M proteins with anti-phagocytic activity and some an additional structurally related M –like protein. All of these can bind to various host plasma proteins like fibrinogen, plasminogen, albumin, IgA, IgG and factor H (Greenwood et al., 2007, Madigan et al., 2008).

Binding of fibrinogen to streptococci expressing M proteins blocks complement disposition on the bacterial cell surface (Anand et al., 2012). Factor H, a complement regulatory protein binds to the C repeat region of the M protein, and a factor H-like protein binds to the hypervariable region (Fischetti et al., 1995). These 2 events both inhibit the deposition of soluble opsonic C3b to the bacterial surface and the net effect is inhibition of opsonisation by the alternate complement pathway (Fischetti et al., 1995). GABHS can release some of the M proteins with its own cysteine protease (Raeder et al., 1998). If shed in the circulation the protein forms complexes with fibrinogen which by indirect pathways induces the release of inflammatory mediators from neutrophils. This process plays an important part in the leakage of plasma into the tissues and lungs (Lamagni et al., 2008).

1.3.3 Anti-streptococcal antibodies as surrogate markers of infection

Due to the delayed clinical manifestations in some of the post-streptococcal syndromes identification (culturing) and serotyping of the GABHS is not always possible as the infection may have cleared. In addition to a clinical history of pharyngitis or skin infection, surrogate markers can be used instead to assess recent infection. Surrogate markers measure the concentrations of host antibody levels against the extracellular antigens of GABHS. An increase or decrease in the antibodies can give some indication of the course of infection (acute, chronic, relapse or recovery) and hence the indication for treatment.

1.3.3.1 Anti-Streptolysin O titre (ASOT)

Neutralising antibodies are produced by the host as a protective measure against SLO and are termed anti-streptolysin O (ASO) antibodies (Todd, 1932; Wannamaker, 1958). Antibody titres against SLO (ASOT) rise during GABHS infection and usually peak between 3 and 6 weeks after acute infection before falling (Todd, 1932). ASOT values where there is no clinical suspicion of rheumatism are less than 200 units in adults and less than 300 units

in children. Upper limit of normal ASOT values are known to vary with respect to site of infection, geographical locations and even season (Shet and Kaplan, 2002). A raised ASOT has been observed in 64% of children with TS as compared to 15% of paediatric neurological disease controls ($p < 0.0001$) (Church et al., 2003). Measurement of ASOT has its limitations, since over diagnosis of acute rheumatic fever (ARF) based on a raised ASOT is not uncommon (Kotby et al., 2012).

1.3.3.2 Anti-deoxyribonuclease B titre (Anti-DNase B)

Streptococcus also produces a number of deoxyribonucleases (DNases A, B, C and D) which can break down deoxyribonucleic acid (DNA) and also elicit an immune response (Ayoub et al., 1962; Wannamaker., 1958). Due to the limitations of ASOT, measuring antibodies against DNase is also a useful indicator of GABHS as it is raised in skin infections and is elevated for longer than ASOT (Ayoub et al., 1962). Neutralising antibodies against DNase B rather than A, C or D result in the strongest response during streptococcal infections (Wannamaker, 1959). Normal range of anti-DNase B considered by most laboratories in the United Kingdom is <300 IU/mL. The benefit of anti-DNase B is that the antibody response is still detectable later on in the disease course compared to ASOT and is more pronounced (Tiesler et al., 1976). Anti-DNase titres are not thought to reach their maximum titre until 6 to 8 weeks after acute infection (Kaplan et al., 1974). Other surrogate markers exist such as anti-hyaluronidase, although it closely mirrors that of anti-DNase B and adds little to the diagnostic utility of the anti-DNase B test (Hederstedt et al., 1978).

1.3.4 Molecular Mimicry Model

Acute GABHS infections may take the form of pharyngitis, scarlet fever, cellulitis, or impetigo. Invasive infections can result in streptococcal toxic shock syndrome necrotizing fasciitis and myositis (Cunningham, 2000, Cunningham, 2008). Patients may also develop immune-mediated sequelae such as acute rheumatic fever and acute glomerulonephritis (Cunningham, 2000; Cunningham, 2004). *S. agalactiae* may cause meningitis, neonatal sepsis, and pneumonia in neonates; adults may experience vaginitis, puerperal fever, urinary tract infection, skin infection, and endocarditis (Lindahl et al., 2005).

GABHS express a number of cell surface components and extracellular products that play an important role in its pathogenesis and in the immune response of the host as discussed previously. Although the pathophysiology of post-streptococcal disorders is not completely understood, antigenic mimicry is proposed to be the triggering cause of autoimmunity (Hahn et al., 2005, Greenwood et al., 2007)

Evidence suggests that GABHS contain antigens which are similar to host antigens and can stimulate B and T cells to respond to self (Cunningham, 2000). The streptococcal M protein, as well as peptidoglycan, N-acetylglucosamine, and group-specific carbohydrate, contain antigenic epitopes that mimic those of mammalian muscle and connective tissue (Bordeur, 2003). Of all the streptococcal antigens involved in molecular mimicry, the M protein has been best investigated and characterized (Cunningham, 2000; Tewodros and Kronvall, 2005). Opsonophagocytosis, mediated by serum opsonins, such as antibodies or complement, is a key mechanism for host protective immunity against streptococcal infection (Baker et al., 1982). M protein has been shown to contribute to virulence, conferring resistance to phagocytosis under non-immune conditions by interfering with the complement pathway (Carlsson et al., 2005) .

The pathogenic response by a susceptible host most likely involves induction of cross-reactive autoantibodies and the recognition of microbial epitopes by the major histocompatibility complex (MHC) proteins which present these epitopes to inflammatory (Th_1 , $CD4^+$) and cytotoxic ($CD8^+$) T cell subsets (Cunningham, 2004). In studies to understand the mechanisms by which streptococcal antigens lead to autoimmune and post-infectious sequelae, production of human monoclonal antibodies and T cell clones have been utilized (Ellis et al., 2005, Kirvan et al., 2006)

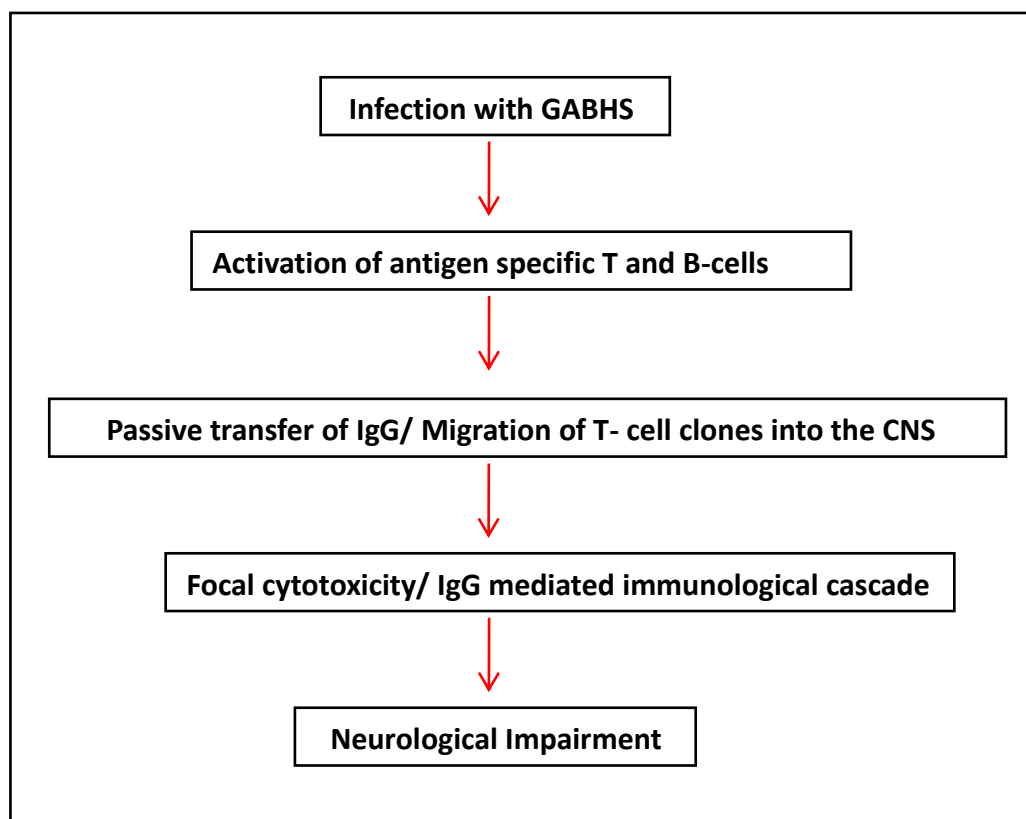


Figure 1.6: Molecular Mimicry Model. The current molecular mimicry hypothesis for the pathology of PANDAS is that streptococcal infection occurring in a vulnerable host leads to production/activation of immune cells that cross-react with the cellular components in the CNS eventually leading to neurological impairment.

1.4 POSTULATED POST-STREPTOCOCCAL DISORDERS

An understanding of the diverse nature of disease complications attributable to this organism is an important cornerstone of paediatric medicine. The following sections briefly detail some of the prevalent clinical entities in which molecular mimicry of GABHS is believed to lead to immunopathogenic tissue damage even after the successful elimination of the eliciting organism.

1.4.1 Acute Rheumatic Fever (ARF)

1.4.1.1 Background

Acute rheumatic fever (ARF) is an autoimmune inflammatory process that develops as sequelae of GABHS infection (PubMed Health; Carapetis et al., 2005). ARF is characterized by non-suppurative inflammatory lesions of the joints, heart, subcutaneous tissue and CNS (Madden and Kelly, 2009). It is most prevalent in children between 5 and 15 years of age, although it can also develop in adults (Cunningham, 200; Carapetis et al., 2005). The recurrence of rheumatic fever is relatively common in the absence of maintenance of low dose antibiotics, especially during the first three to five years after the first episode. Heart complications may be long-term and severe, particularly if valves are involved (Carapetis et al., 2005). Disease symptoms include abdominal pain, fever, cardiac problems which may not have symptoms, or may result in shortness of breath and chest pain, joint pain and swelling, arthritis, nosebleeds, skin nodules, skin rash and Sydenhams chorea (emotional instability, muscle weakness and quick, uncoordinated jerky movements that mainly affect the face, feet, and hands) (Binotto et al., 2002; Carapetis et al., 2005).

1.4.1.2 Pathophysiology

Molecular mimicry between streptococcal and human proteins has been proposed as the triggering factor leading to autoimmune humoral and cellular responses towards the human tissue in rheumatic fever and rheumatic heart disease (RHD) (Hahn et al., 2005, Guilherme et al., 2005). Streptococcal M protein has been found to be structurally and functionally similar to the most prominent of heart antigens cardiac myosin. Cross reactivity between the streptococcal M protein and cardiac myosin have been observed using affinity purified antibodies from ARF sera or sera from rabbits immunized with streptococcal pep M-5 protein (Cunningham et al., 1988). Results indicate three M5 regions that cross react with heart protein fractions, residues 1-25, 81-103 and 163-177 (Cunningham et al., 1988).

Autoantibodies against the heart were associated with RF by Cavelti in 1943. The first indication that anti-heart antibodies cross reacted with GABHS was found in 1963 by Kaplan et al. (1963) when they showed that anti-heart antibodies present in the human sera could be absorbed by group A streptococci or their cell wall (Kaplan, 1963). The role of cross-reactive and polyspecific antibodies in the pathogenesis of RF is not yet clear, however two monoclonal antibodies (MAb) namely human MAb 1.H9 and mouse MAb 36.2.2 were found to be cytotoxic to heart cells in culture in the presence of complement (Adderson et al., 1998). Various studies have also investigated T cell responses to streptococcal M proteins. T cells with high affinity for self-antigens are normally removed from the repertoire during intrathymic deletion; those that escape to the periphery most likely have low to intermediate affinity for self-antigens. These T cells are subject to peripheral tolerance control but may be activated under certain conditions and subsequently contribute to autoimmune disease (Ellis et al., 2005). Cytotoxic T cells were

found to be stimulated by M protein with their presence detected in the blood of patients with ARF (Dale and Beachey, 1987, Cunningham, 2004). In a study by Gorton et al. (2009) rats immunized with streptococcal M5 protein developed valvular lesions, distinguished by infiltration of CD3⁺, CD4⁺, and CD68⁺ cells into valve tissue, consistent with human studies that suggest that RF/RHD are mediated by inflammatory CD4⁺ T cells and CD68⁺ macrophages (Gorton et al., 2009)

1.4.1.3 Treatment

Management and prevention of ARF includes: Treatment of the GABHS infection that is the identified cause of the disease with intramuscular penicillin G benzathine, oral penicillin V potassium, and oral amoxicillin (Brook and Doah, 2006; Armstrong, 2009). Anti-inflammatory agents like salicylates and steroids are used to control the arthritis, fever, and other acute symptoms (Kleinerman et al., 1981), Intravenous immunoglobulin (IVIG) is also performed although it has not been shown to reduce the risk of rheumatic heart disease or to substantially improve the clinical course (Voss et al., 2001). Treatment of chorea though IVIG, steroids, and plasmapheresis has have been successfully used in refractory chorea, although conclusive evidence of their efficacy is also limited (Crapetis et al., 2005; Weiner et al., 2007). Bed rest is essential in patients with cardiac involvement. Carditis resulting in heart failure is treated with conventional measures like corticosteroids although digoxin and diuretics are the mainstay of therapy (Feldman and McNamara, 2000; Crapetis et al., 2005).

1.4.2 Sydenhams chorea (SC)

1.4.2.1 Background

Sydenhams chorea is the principle neurological manifestation of rheumatic fever. The symptoms of SC can vary from a halting gait and slight grimacing to involuntary movements that are frequent and severe enough to be incapacitating (Moore, 1996; Dale, 2003). SC results from childhood infection with GABHS and is reported to occur in 20-30% of patients with RF. The disease is usually latent, occurring up to 6 months after the acute infection, but might occasionally be the presenting symptom of RF. SC is more common in females than males and most patients are children, below 18 years of age (Walker and Wilmschurst, 2010). Adult onset of SC is comparatively rare and most of the adult cases are associated with exacerbation of chorea following childhood SC (NINDS, WeMove.org, Moore, 1996). Symptoms of SC include fever, arthritis, carditis, subcutaneous nodules and choreic movements (rapid, uncoordinated jerking movements affecting primarily the face, feet and hands) like deterioration in handwriting, difficulties feeding and walking, facial grimacing and slurred speech (dysarthria) (Dale, 2003, Walker and Wilmschurst, 2010). Several emotional or behavioral disturbances are also associated with SC like attention deficit hyperactivity disorder (ADHD), age-regressed behaviors, frequent mood changes and excessive emotional reactions including uncontrollable crying and transient psychosis (Moore, 1996, Dale, 2003).

1.4.2.2 Pathophysiology

Evidence of molecular mimicry with mechanism of pathogenesis similar to ARF has been proposed in SC. A study demonstrated the presence of cross reactive autoantibodies in human hybridoma cell lines derived from SC patients. These monoclonal antibodies recognized N-acetyl- β -D-glucosamine (GlcNAc), the major epitope of GABHS surface

carbohydrate and were seen cross reacting with lysoganglioside , a central nervous system ganglioside which is known to play a role neuronal signal transduction (Kirvan et al., 2003). Binding of autoantibodies to the brain lysoganglioside activated calcium/calmodulin dependent protein kinase II (CAMKinase II) which is proposed to regulate functions like neurotransmitter synthesis and release. Antibodies to N-acetyl- β -D-glucosamine bound to tissue sections derived from the human basal ganglia (Fujinami and Sweeten, 2003, Kirvan et al., 2007).

Further evidence that SC is a basal ganglia disorder comes through evidence derived from magnetic resonance imaging (MRI) volumetric studies that have shown increased sizes of the caudate, putamen, and globus pallidus in the Sydenham's chorea group with no increase seen in total cerebral, prefrontal or midfrontal volumes or thalamic area (Giedd et al., 1995). Pathological findings by Colony et al. (1956) (Colony and Malamud, 1956) have also revealed specific changes to the basal ganglia, including cellular infiltration and neuronal loss with relative sparing of other brain areas in SC (Church et al., 2002, Dale and Heyman, 2002). These results indicated the selective involvement of the basal ganglia in SC.

In an attempt to gain a more precise understanding of chorea antibody specificities, monoclonal antibodies derived from SC patients were studied for reactivity with brain proteins. These antibodies were found to react with the cytoskeletal α -helical protein tubulin in addition to GlcNAc. Competitive inhibition studies have shown that the tubulin reactivity of the chorea mAbs was blocked by three gangliosides with lysoganglioside GM1 as the potent inhibitor (Kirvan et al., 2007). Anti-GlcNAc autoantibodies from RF and uncomplicated pharyngitis cases have been found to be cross reactive to different cytoskeletal and microbial proteins like human cardiac myosin, epidermal keratin, streptococcal recombinant M6 and pepM5 in contrast to SC mAb which exhibit mAbs which

appear to have unique antigen specificity (Adderson et al., 1998). This difference in the antigen cross reactivity profile may be an indication for the presence of different subsets of anti-GlcNAc mAbs contributing towards the different clinical manifestations of ARF.

1.4.2.3 Treatment

Therapeutic interventions in patients with SC are based on the aetiology, pathology, pathophysiology and clinical presentation. Primary treatment with penicillin is considered mandatory to eliminate the streptococcus (Walker, 2010). Secondary prophylaxis with long-term penicillin is prescribed to protect the heart, however, whether it prevents relapses of SC is still debatable (Berrios et al., 1985). The increased understanding of the pathophysiology of SC has led to the use of agents which affect the neurotransmitters dopamine (e.g. halperidol) (Shenker et al., 1973) and GABA (e.g. benzodiazepines) (Edgar, 2003). Immunomodulatory therapies like corticosteroids, IVIGs and plasma exchange are used to shorten the course of the illness and to prevent complications (Garvey et al. 2005).

1.4.3 Paediatric Autoimmune Neuropsychiatric Disorders Associated with Streptococcal Infections (PANDAS)

1.4.3.1 Background

Swedo and colleagues proposed the existence of subgroup paediatric autoimmune neuropsychiatric disorders associated with streptococcal infections (PANDAS) that included a subset of children with tic disorder and/or obsessive compulsive disorder who have an abrupt onset or exacerbations of symptoms temporally related to streptococcal infection (Swedo et al., 1998). They proposed five diagnostic criteria that needed to be fulfilled for inclusion in the PANDAS subgroup:

- (1) Presence of OCD or a tic disorder
- (2) Childhood onset
- (3) Abrupt onset of symptoms
- (4) Exacerbations of symptoms temporally related to a preceding GABHS infection
- (5) Association of neurological abnormalities including hyperactivity and choreiform movements

Host vulnerability for PANDAS might be the result of a combination of genetic susceptibility, developmental factors and abnormal immune responsivity (Swedo, 2002). Genetic factors may play a greater role in childhood-onset cases as rates of tic disorders and OCD in first-degree relatives of children in the PANDAS subgroup are found to be higher than those in the general population (12% total; 15% in parents; 5% in siblings) (Lougee et al., 2000). Similarly development vulnerability is suggested because of the high rate of disease occurrence among grade school age children with 6-7 years being the peak age for the onset of symptoms (Swedo et al., 1998).

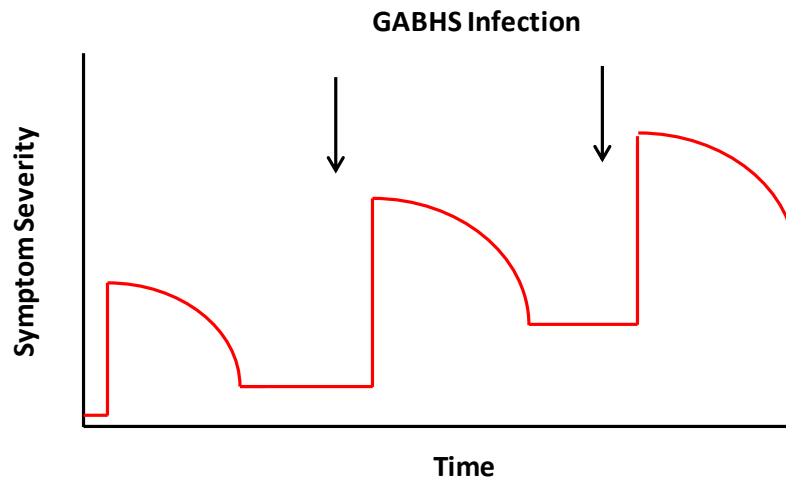


Figure 1.7: Clinical course of PANDAS. The graph above depicts the clinical course of PANDAS with periods of symptom quiescence, followed by exacerbations with abrupt onset and gradual resolution. Neuropsychiatric exacerbations in PANDAS begin at the time of GABHS infection or within one to two weeks after infection.

1.4.3.2 Pathophysiology

Most of the knowledge about PANDAS has been obtained by studying patients with a known tic disorder or long-standing OCD (Murphy et al., 2010). Neuroimaging studies have revealed an increased basal ganglia volumes and the proposed mechanism involving the cross-reaction of streptococcal antibodies with basal ganglia tissue (Murphy et al., 2010). The hypothesis of a possible involvement of the immune system seems justified from quantitative alterations of TNF-alpha, IL-6 and IL-1 in the patients' serum with such syndromes (Marconi et al., 2009). However, the role of autoimmunity in PANDAS has been controversial. Reports of response to treatment with immune-modifying therapies, such as glucocorticoids, IVIG, and plasma exchange have supported for a role of autoimmunity (Allen et al., 1995; Perlmutter et al., 1999). However, the response to plasma exchange appears to be specific for children with OCD or a tic disorder with a preceding GABHS

infection as in an open trial it provided no benefit for children with OCD whose symptom exacerbation did not follow GABHS infection (Nicolson et al., 2000).

An autoimmune mechanism for PANDAS is also supported by studies demonstrating increased anti-neuronal antibodies in patients diagnosed with PANDAS as compared to patients with OCD or tic disorders but no evidence of GABHS infection and patients with GABHS infection but without OCD or tic disorder (Church et al., 2003; Hoffmann et al., 2004). However some other studies looking at autoantibody profiles have not been able to distinguish patients with PANDAS or TS from control patients (Singer et al., 2005; Morris et al., 2009). Methodologic differences might explain the conflicting results of these studies (Rippell et al., 2005). Failure of microinfusion of PANDAS serum into rodent striatum to produce behavioural changes (Loiselle et al., 2004) and failure of immunologic markers to correlate with clinical exacerbations in PANDAS patients in a longitudinal case-control study (Singer et al., 2008) provide some conflicting evidence against the hypothesis that PANDAS is caused by anti-neuronal antibodies (Kaplan et al., 2011).

1.4.3.3 Treatment

The management of PANDAS centres on providing standard therapies for GABHS infection and OCD/tic disorder. There have been no randomized controlled trials of antibiotic treatment in children suspected of having PANDAS. In a prospective study that identified 12 children with abrupt onset of neuropsychiatric symptoms and evidence of recent GABHS infection, anti-streptococcal therapy using penicillin or cephalosporin was associated with prompt symptom resolution in all cases (Murphy et al., 2002). Immunomodulatory therapies include glucocorticoids, plasma exchange, and intravenous IVIG provided PANDAS is an autoimmune disorder as this still remains controversial.

1.4.4 Tourettes syndrome (TS)

1.4.4.1 Background

Gilles de la Tourette's syndrome (TS) is a neuropsychiatric disorder characterised by the presence of motor and phonic tics (Robertson MM, 2000; Leckman, 2002; Church et al., 2003). A tic is an involuntary, repetitive, rapid, sudden, non-rhythmic, stereotyped movement or sound, which begins in childhood or early adolescence (Hoekstra et al., 2004). They occur in bouts though the day and wax and wane in severity over time, changing in frequency, number and complexity (Robertson, 2000; Leckman, 2002). Symptoms begin in children between the ages of 3 and 8 years, usually with simple motor tics (Leckman, 2002). These involve only 1 muscle group and include movements such as eye blinking and head jerking (Singer, 2005). More complex motor tics involve a co-ordinated sequence of movements or group of simple movements such as body or facial contortions, smelling, licking, spitting, jumping, touching, squatting and echopraxia (imitating other people's actions) (Robertson, 2000; Singer, 2005). The most extreme forms of TS also involve forceful bouts of self-harmful actions including hitting and biting but these only occur mainly in adults (Robertson, 2000). Phonic tics develop later in patients with a mean onset of 11 years, although some cases have reported them in children as young as 3 years old (Robertson, 2000; Leckman, 2002). Simple phonic tics include sniffing, throat clearing, barking and grunting. Complex expressions include syllables, palailia (repeating one's own words), echolalia (repeating words of others) and coprolalia (uttering obscene words) (Singer, 2005). These occur in a substantial number of patients except for coprolalia which is only present in 10% of patients with a mean onset of 15 years (Robertson, 2000; Singer, 2005). Tics can be fast and brief with an abrupt onset (clonic) or sustained and slow (dystonic) (Leckman, 2000). Studies have shown that TS has a prevalence of 5 per 10,000

and is more common in males. About 50% of patients do not express tics by 18 years of age and in adulthood tics gradually become less severe (Church et al., 2003).

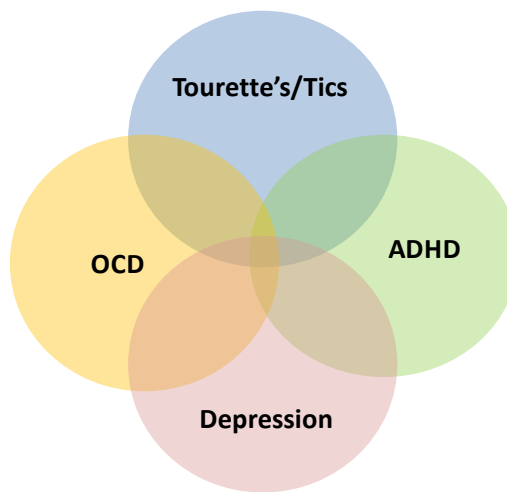


Figure 1.8: Tourette's syndrome and related disorders. A substantial proportion of TS patients have comorbid neuropsychiatric disorders including obsessive compulsive disorder (OCD), attention deficit hyperactivity disorder (ADHD) and depression (Robertson, 2002; Church et al., 2003). It has been suggested that TS and OCD are actually part of the same disease spectrum (Church et al., 2003).

1.4.4.2 Pathophysiology

The aetiology of TS is unknown but it is proposed that both genetic disposition and environmental factors are involved (Garvey et al., 1998; Church et al., 2003). There is little doubt that TS has a genetic background but the precise inheritance pattern has yet to be determined. Preliminary studies have suggested an autosomal dominant inheritance and genome screening has revealed two regions, 4q and 8p with increased lod scores. It is proposed that TS susceptibility genes might be located at these loci (Church et al., 2003).

Basal ganglia involvement in TS is supported by the presence of ABGA in TS patients. One study reported a correlation between ASOT and ABGA and observed elevated titres in 91%

of ABGA-positive patients and in 57% of ABGA-negative patients (Church et al., 2003). They concluded that for some TS patients the presence of ABGA was related to GABHS infections. The surprising result however, was the relatively high prevalence of GABHS in ABGA-negative patients, which suggested ABGA reactivity could wax and wane similar to clinical symptoms. This hypothesis was supported by follow-up clinics 3 months later, where 3 out of 4 patients who were previously ABGA negative, were now ABGA positive with reduced ASOT (Church et al., 2003).

Other studies disagree about the significance of ABGA in TS and OCD patients and claim healthy subjects can also have relatively high anti-neuronal titres (Hoekstra et al., 2003). One particular study did detect a relationship between ABGA and an 83 kDa antigen in TS-OCD patients but also suggested that these antibodies were not a direct consequence of GABHS infection alone (Pavone et al., 2004). Another explanation for ABGA in SC, TS and OCD is that antibodies are produced due to an “epiphenomenon secondary to basal ganglia damage” (Hoekstra et al., 2003). This theory however is thought unlikely as studies have shown patients with inflammatory, ischemic and metabolic basal ganglia diseases rarely produce ABGA (Church et al., 2003). In addition, tics have also been observed in rats after IgG from TS patients was micro infused into the brain striatum, further supporting a pathological role for ABGA (Taylor et al., 2002; Church et al., 2003).

Apart from basal ganglia dysfunction the other source abnormality in TS is thought to be inappropriate regulation of neurotransmitters, especially dopamine (Singer et al., 1991). Strong evidence has indicated dopamine excess or supersensitivity of the postsynaptic dopamine receptors as the underlying pathophysiological mechanism of TS (Singer et al., 1982; Singer et al., 1998). Another hypothesis of dysfunction is a neurophysiologic deficit secondary to neurotransmitter abnormalities that results in failure of inhibition of the

frontal-subcortical motor circuits an area that has prominent interconnections with the basal ganglia (Singer, 1997; Bagheri et al., 1999).

1.4.4.3 Treatment

The management of TS is multifaceted with the treatment primarily aimed at frequent or disabling tics, coexisting behavioural symptoms, and patient/family education. The alpha2-adrenergic drugs clonidine and guanfacine both of which decreases plasma norepinephrine levels are first-line agents in treating mild to moderate tics (Bagheri et al., 1999; King et al., 2005). Neuroleptics which block the D2 dopamine receptor e.g. haloperidol and pimozide are the most effective medications for treating tics, and are used by many experts (Guilliano et al., 1998; Bagheri et al., 1999). However, their side effect profile, which includes extrapyramidal symptoms and tardive dyskinesia, limits their use as first-line therapy (Guilliano et al., 1998; Bagheri et al., 1999). More recently atypical neuroleptics that interact with both serotonin and dopamine receptors have been shown to be effective and have less extrapyramidal side effects. Within this group risperidone has shown efficacy equal to that of clonidine (Bruun and Budman, 1996; Scahill et al., 2003). Furthermore, botulinum toxin-A has been effective in treating motor and vocal tics in some patients (Aguirregomez et al., 2008). Additionally anteromedial globus pallidus interna deep brain stimulation (DBS) is found to be an effective and well-tolerated treatment for a subgroup of patients with severe TS (Bajwa et al., 2007; Cannon et al., 2012). Selective serotonin reuptake inhibitors (SSRIs) and clomipramine are predominantly used to treat OCD symptoms (Kellner, 2010).

1.4.5 Dystonia

1.4.5.1 Background

Dystonia is a movement disorder characterized by directional, patterned and sustained muscle contractions leading to repetitive twisting movements, and abnormal postures of the trunk, neck, face, or arms and legs (Stamelou et al., 2011, Fahn, 1987). Neuroimaging studies have provided insight into the systems-level disturbances that are responsible for dystonia which indicate a central role for abnormal plasticity leading to distortion of sensory fields in the sensorimotor cortex and abnormal signalling in the basal ganglia (Breakefield et al., 2008). Current treatment options of dystonia rely on drugs acting on cholinergic, dopaminergic and γ -aminobutyric acid (GABA)ergic receptors (Breakefield et al., 2008). Although considered a third-line treatment certain forms of dystonia are known to respond well to deep brain stimulation of the globus pallidus, indicative of the fact that movement abnormalities are potentially reversible (Krauss, 2007).

In the recent years there has been a rationalisation of the classification of dystonia and a much deeper understanding into the causes through the study of genetics, neurophysiology, and functional imaging (Phukan et al., 2011). Dystonia has been classified based on topographic distribution, age of onset, aetiology and genetics (Tarsy and Simon, 2006; Donaldson et al., 2012). Topographic classification of dystonia includes: focal (single region), segmental (two or more adjacent regions), multifocal (two or more nonadjacent regions), generalized (leg or legs, trunk and one other region) and hemidystonia (ipsilateral arm and leg) (Geyer and Bressman, 2007). Classification according to the age of onset includes early onset dystonia (≤ 25 years) and late onset dystonia (>25 years). Age of onset is closely related to anatomical distribution early onset dystonia usually progresses from focal limb to severe generalized form as opposed to late onset which remains localized or

segmental, and is usually not progressive (Berardelli et al., 1998; Bressmann, 2004). Genetically determined dystonias is a rapidly evolving group based on the loci of genes involved. Loci DYT1 through DYT13 include autosomal dominant, autosomal recessive and X-linked causes of primary dystonia and dystonia-plus syndromes (Doheny et al., 2004). Etiological classification of dystonia includes primary, secondary, paroxysmal and dystonia plus syndromes (Schneider and Bhatia, 2010, Fahn et al., 1998). Primary dystonia occurs in patients who have no signs of structural abnormality in the central nervous system (CNS) (Berardelli et al., 1998). The precise cause of primary dystonia is unknown hence it was originally described as 'idiopathic' as there was no neurophysiological, neurochemical or pathological clues to the underlying aetiology, but many are now known to have a genetic basis (Nemeth, 2001; Donaldson et al., 2012). Cervical dystonia is known to be the most common primary dystonia (Defazio et al., 2004). Secondary dystonia on the other hand is associated with an inherited neurological disorder or exogenous insult. Dystonia-plus syndrome is a sub-category of secondary dystonia in which dystonia is accompanied by a neurological condition but as in primary dystonia there is no neuropathological evidence (Fahn et al., 1998). Lastly paroxysmal dystonias are rare disorders characterized by episodic dystonia and other involuntary movements without symptoms or neurologic findings between episodes. They bear an uncertain relation to other dystonias as they clinically overlap with other episodic disorders such as epilepsy and ataxia (Tarsy and Simon, 2006).

1.4.5.2 Pathophysiology

Most cases of dystonia do not have a specific demonstrable cause. Primary dystonia is often inherited (Geyer and Bressman, 2007). Neurophysiological studies of patients with dystonia and animal models have shown abnormalities of cortical and basal ganglia function (Berardelli et al., 1998). Basal ganglia abnormality could be a result of tumour,

stroke, oxygen deprivation, drug reaction or infection (Geyer and Bressman, 2007). Although the classic post-streptococcal neuropsychiatric disorder is Sydenham chorea recent studies have suggested that the spectrum of postulated post-streptococcal CNS disease is broader, including hyperkinetic movement disorders like dystonia and tics (Dale, 2003; Karagulle-Kendi et al., 2008). Infantile bilateral striatal necrosis (IBSN) is an abnormality of the basal ganglia associated with acute dystonia in children is also known to fall under the post-streptococcal autoimmune neuropsychiatric spectrum (Dale et al., 2002; Karagulle-Kendi et al., 2008). Additionally, there are high rates of behavioural sequelae, particularly that of psychiatric disorders such as OCD, anxiety and depression. All these findings have led to the hypothesis that similar immune-mediated basal ganglia processes may be operating in all these neuropsychiatric disorders (Dale, 2003).

1.4.5.3 Treatment

Pathogenesis-targeted treatment is still elusive however currently available symptomatic treatment strategies are fairly effective for certain types of dystonia. Botulinum toxin A (or type B if there is resistance to type A) is regarded as first-line treatment for primary cervical dystonia and blepharospasm and is probably effective in other types of upper limb dystonias (Costa et al., 2005). However it is not licensed for use in children. Drug treatments for dystonia include levodopa (Hwang et al., 2001), anti-cholinergic agents and tetrabenazine/benzodiazepines (Marino et al., 1993; Albanese et al., 2006). The absolute and comparative efficacy and tolerability of anticholinergic agents is found to be poorly documented in children and there is no proof of their efficacy in adults either (Albanese et al., 2006). Pallidal DBS is found to be very effective in primary generalized or segmental dystonia (Kupsch et al., 2006; Haridas et al., 2011). It is found to be less effective in secondary dystonia with the exception of tardive dystonia (Kupsch et al., 2006). Intrathecal

baclofen has been employed in patients with severe generalized dystonia particularly the ones with concomitant severe spasticity (Albanese et al., 2006).

1.4.6 Encephalitis Lethargica (EL)

1.4.6.1 Background

Encephalitis lethargica also known as sleeping sickness was first described by neurologist Constantin von Economo in 1917 (Dourmashkin, 1997). An epidemic of the disease swept around the world between 1915 and 1926. The disease is highly polymorphic with fluctuating signs and symptoms however prodromal symptoms include headache, double vision, high fever, delayed physical and mental response, and lethargy (Triarhou LC, 2006). In acute cases, patients may enter coma-like state termed akinetic mutism (Triarhou, 2006). They may also experience Parkinsonism, abnormal eye movements, upper body weakness, tremors, and behavioural changes. Pathological findings have localized the encephalitis to basal ganglia and midbrain structures (Triarhou, 2006, Vilensky et al., 2007).

1.4.6.1 Pathophysiology

Although the exact cause of EL is still unknown it is postulated to be an autoimmune disease. Because the EL epidemic occurred around the same time as the 1918 influenza pandemic the two were thought to be related (Ravenholt and Foege, 1982). However, the failure to find the identified influenza ribonucleic acid (RNA) in the archived EL brains dismissed this association (Taubenberger et al., 1997, McCall et al., 2008). However a recent study has found 27 nm virus-like particles (VLP) in the cytoplasm and nuclei of midbrain neurons in four classical EL cases. Additionally large 50nm VLP and 27nm intranuclear VLP were observed in two modern EL cases and a post-encephalitic

Parkinsonism (PEP) CASE. The VLPs were identified as enterovirus by sequence analysis (Dourmashkin et al., 2012).

All the recent sporadic cases of EL-like syndrome have presented with pharyngeal infections. GABHS being the most common cause of pharyngitis and a recognized cause of immune mediated basal ganglia dysfunction in SC (Church et al., 2002) has hence, been implicated in EL. It is hypothesized that antibodies induced after GABHS infection cross-react with components of the basal ganglia, resulting in movement and psychiatric disorders (Dale et al., 2004). A study by Dale et al. examining 20 cases of EL was able to demonstrate autoantibodies reactive against discrete basal ganglia autoantigens in 95% of the patients. Rather than polyspecific binding, reactivity was seen against several common basal ganglia antigens 40, 45 and 60 and 98 kDa (discussed later). Out of these the first three have are important Sydenham's neuronal antigens but the 98 kDa antigen was novel to the EL phenotype. The CSF examination confirmed that the autoantibodies are present in the CNS, although whether these antibodies are mainly produced intrathecally or peripherally (with transfer into the CNS) has not been examined (Dale et al., 2004).

1.4.6.3 Treatment

Treatment of EL is symptomatic. There is little evidence so far of a consistent effective treatment for the initial stages, though some patients given steroids have seen improvement (Blunt et al., 1997). Levodopa and other anti-parkinson drugs often produce dramatic responses in people with this condition (McAuley et al., 1999). However, in most of the patients who were given L-DOPA in the 1960s, the amelioration of the disease was short lived (McAuley et al., 1999). Some other drugs used to treat EL post 1940 included neuroleptics, anti-cholinergics, anti-depressants, e.g. imipramine, corticosteroids and

antibiotics. However the efficacy of these drugs for EL is still controversial due to insufficient or contradictory data (Vilensky, 2011).

1.4.7 NMDAR Encephalitis

1.4.7.1 Background

The *N*-methyl-D-aspartate receptor (NMDAR) is a specific type of ionotropic receptor belonging to the glutamate receptor superfamily together with α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate receptors (Dingledine et al., 1999). Both NMDA and non-NMDA receptors are activated by the endogenous transmitter, L-glutamate, however, L-aspartate, a putative endogenous transmitter appears to activate NMDA only (Patneau and Mayer, 1990). NMDAR are inactive at resting membrane potential this is due to a voltage dependent block of the pore by magnesium ions (Mayer et al., 1984) and their activation is secondary to kainate or AMPA receptor activation that depolarises the neuron leading to the release of the Mg⁺ blockade (Ascher and Nowak, 2009). The NMDAR is most abundant in the cortex, basal ganglia, and sensory pathways of the nervous system, but have also been identified in a variety of non-neuronal and peripheral locations (Bolton and Paul, 2006).

The NMDAR have been associated with a wide variety of neurological disorders including ischemic brain damage, epilepsy and more speculatively, neurodegenerative disorders such as Parkinsons, Alzheimers, Huntingtons chorea, and amyotrophic lateral sclerosis (ALS) (Waxman and Lynch, 2005; Ghasemi and Schachter, 2011).

1.4.7.2 Pathophysiology

Anti-NMDA receptor encephalitis has been recently identified as treatment responsive encephalitis associated with anti-NMDA receptor antibodies (Iizuka and Sakai, 2008). These antibodies bind to the NR1/NR2 heteromers of the NMDA receptors and antibody levels are known to correlate with the clinical severity of the disease (Irani and Vincent, 2011). Recent studies have demonstrated that these antibodies cause a reversible reduction in the numbers of cell-surface NMDA receptor clusters in postsynaptic dendrites, suggesting antibody mediated decreased NMDA receptor function (Iizuka, 2009).

NMDAR encephalitis is highly characteristic occurring in five stages: the prodromal phase with viral infection like symptoms, psychotic phase, unresponsive phase, hyperkinetic phase, and gradual recovery phase (Iizuka et al., 2010). Psychiatric manifestations are observed that upon progression lead to decrease of verbal output, catatonia, seizures, dyskinesias, hypoventilation and frequent autonomic instability (Florence et al., 2011). In some cases it is a paraneoplastic syndrome usually affecting childbearing-age female with ovarian tumors, however, recent reports suggest a much higher incidence of nonparaneoplastic cases in males and children (Lebas et al., 2010; Hung et al., 2011).

Clinical features seen in EL coincide with the NMDA receptor encephalitis which might make it a part of the spectrum of anti-NMDAR disorders. Dale et al. (2009) studied sera from paediatric patients with contemporary EL and found 10 sera out of 20 (from 2 males and 8 females, aged 1.3–13 years) and 6/6 cerebrospinal fluid samples positive for antibodies against the NMDA receptor. Different disease phenotypes were observed between the NMDA receptor positive and negative patients suggestive of different pathogenic targets. The antibody concentration in all cases was found to be higher in the serum than CSF (Dale et al., 2009).

1.4.7.3 Treatment

Treatment of anti-NMDAR encephalopathy includes immunotherapy and/or tumour removal. Anecdotal reports have shown that recovery might be spontaneous without tumour resection , however, tumour resection together with aggressive immunotherapy facilitates early functional recovery and 65% of patients with anti-NMDAR encephalopathy exhibit full or near-full recovery (Suzuki et al., 2008). Most patients receive IVIG, plasma exchange or corticosteroids as first-line of immunotherapy (Dalmau et al., 2011). It is likely that patients who don't respond to one form of immunotherapy might respond to other regimens like plasmaphoresis, cyclophosphamide, and rituximab (Irani and Vincent, 2011).

1.5 CANDIDATE AUTOANTIGENS

It has been identified that patients presenting with an encephalitic illness with a subsequent clinical course reminiscent of EL with ABGA and evidence of recent streptococcal infection (Dale et al., 2001; Dale et al., 2004a). ABGA is shown to recognise four main protein bands of 40kDa (aldolase C), 45kDa (neuron specific gamma enolase), 45 kDa (non-neuronal alpha enolase) 60 kDa (pyruvate kinase M1) and 98 kDa (neuron specific alpha/gamma dimer) which have been purified and successfully identified using 2-D gel electrophoresis and mass spectrometry (Dale et al., 2006). All the above antigens are glycolytic enzymes and are involved in energy homeostasis and as expected are found in the cytosol. However, these proteins have also been shown to be located on the neuronal surface (Lim et al., 1983; Nakajima et al., 1994), where they appear to have “moonlighting” or have alternative functions (explained below). These glycolytic enzymes/putative autoantigens have probable roles in energy metabolism, neurotransmission, cell health, maintenance of ion channels, and the control of cellular metabolism. All these roles are plausible and it is possible that autoantibodies could interfere with these roles either directly or indirectly (i.e. via a cell mediated response).

1.5.1 Aldolase C (Ald C)



Glycolytic enzyme aldolase (ald) catalyzes the production of glyceraldehyde phosphate from fructose 1, 6-bisphosphate. Aldolase has 3 isoforms: aldolase A is expressed largely in the muscle, aldolase B in the liver and aldolase C in the brain and neuroendocrine cells (Buono et al., 2001). Ald C mRNA is greatest in cerebellar neurons but is found to a lesser degree albeit in the striatum, medulla and occipital lobe (Ignaki et al., 1988).

Aldolase may have functions beyond glycolysis and has been implicated in the functioning of Vacuolar proton- translocating ATPases (V-ATPases). These are a family of highly conserved proton pumps that couple hydrolysis of cytosolic adenosine triphosphate (ATP) to proton transport out of the cytosol (Forgac, 1999). Residing on the intracellular membrane these pumps are central to pH homeostasis in organelles. Some cells express V-ATPases at the plasma membrane, where they carry out cell-specific functions such as renal acidification, bone reabsorption, homeostasis of cytoplasmic pH and sperm maturation (Forgac, 2007). The direct binding interaction between V-ATPase and aldolase might be a new mechanism for the regulation of the V-ATPase and may underlie the proximal tubule acidification defect in hereditary fructose intolerance (Lu et al., 2001).

Ald C gene has been identified as a candidate hypoxia-regulated gene in mouse lung epithelial (MLE) (Jean et al., 2006). Aldolase associates with the actin cytoskeleton and can provide a local source of ATP production that is important for cytoskeletal integrity (Schindler et al., 2001). In addition, ald C has been found in tight association with a plasma membrane oxidoreductase complex in brain cells. The plasma membrane oxidoreductase complex is believed to function as an extracellular membrane redox sensor that induces cellular responses to external oxidant stress (Bulliard et al., 1997).

It is the C isoform of aldolase that has been identified as one of the putative autoantigens in post-streptococcal disorders. Aldolase C-positive cerebellar Purkinje cells are observed to be resistant to delayed death after cerebral trauma and AMPA-mediated excitotoxicity which highlights its role in neuroprotection (Slemmer et al., 2007). Glucose metabolism has been indicated to play an important role in the pathogenesis of rheumatoid arthritis (RA) and evidence indicates that RA synovial tissues have increased glycolytic activity which leads to an acidic microenvironment that further induced the transformation of normal

synovial cells. Enolase and aldolase are the two key enzymes of the glycolysis pathway that are observed to promote RA autoimmunity by acting as autoantigens (Goëb et al., 2009; Chang and Wei, 2011). Furthermore, Ald A has been identified as a candidate autoantigens in Alzheimer's disease with sera IgG of >50% of 45 AD patients binding to it. However, immunization of rats and mice with Ald A in complete Freund's adjuvant was found not to be pathogenic (Mor et al., 2005). Significantly higher autoantibodies against Ald B have been determined in sera of patients with chronic hepatitis and liver cirrhosis as compared to healthy subjects with anti-aldolase B antibody titer indicating the severity of liver dysfunction (Maniratanachote et al., 2005).

1.5.2 Enolase (Eno)



The enzyme enolase also known as phosphopyruvate dehydratase was discovered by Lohmann and Meyerhof in 1934 (Lohman.K 1934). It is responsible for the catalysis of 2-phosphoglycerate (2-PG) to phosphoenolpyruvate (PEP), the penultimate step of glycolysis.

The structure of enolase is highly conserved with a homology of 40-90% between different species (Pancholi V, 2001). There are three subunits of enolase namely α , β , and γ , each encoded by a separate gene that can combine to form five different isoenzymes: $\alpha\alpha$, $\alpha\beta$, $\alpha\gamma$, $\beta\beta$, and $\gamma\gamma$. In mammals there is 82% homology between the amino acid sequences of α -, β - and γ -enolase monomers (Witkowska et al., 2005). Alpha-enolase (also called non-neuronal enolase) is ubiquitously expressed in the early stage of embryonic development, beta-enolase is expressed in adult skeletal and cardiac muscles, and gamma-enolase (also called neuron-specific enolase (NSE)) is expressed in mature neuron and neuroendocrine cells (Marangos and Schmechel, 1987, Deloulme et al., 1997). Using sensitive immunoassays,

significant levels of NSE have been found in differentiated tissues other than nervous tissues (Haimoto et al., 1985).

Enolase apart from being involved in glycolysis has additional roles as plasminogen binding receptor, heat shock protein, Myc-binding protein, eye τ -crystallin protein, and an endothelial stress protein. It acts as a plasminogen receptor on neuronal membranes mediating interactions between microglia and dopaminergic neurons (Nakajima et al., 1994). The enolase binding to plasminogen induces plasminogen transformation into plasmin and is thought to be a virulence factor by preventing the generation of fibrin clots and thus enabling tissue invasion, as described for *S. pyogenes* and *S. Pneumonia* (Fontan et al., 2000). NSE can form complexes with other glycolytic enzymes (e.g. aldolase C) which monitor oxidative stress levels at the cell surface (Bulliard et al., 1997). In addition, α -enolase when located in the nucleus is a Myc-binding protein (MBP-1) playing a vital and crucial role in cell growth and differentiation (Terrier et al., 2007).

Studies have reported the presence of enolase-specific autoantibodies in a range of psychiatric, degenerative and inflammatory disorders, including discoid lupus erythematosus, autoimmune polyglandular syndrome type 1, primary and secondary membranous nephropathy, cancer-associated retinopathy, autoimmune hepatitis type 1, mixed cryoglobulinemia with renal involvement, cystoid macular oedema and endometriosis (Gitlits et al., 2001). In addition, autoantibodies which cross react with GABHS and human α -enolase have been observed ARF patients (Fontan et al., 2000). In recent study involving anti-optic nerve autoantibodies, 55% of 209 patients showed specific neuronal autoantibodies. Some of the major antigenic targets for these antibodies were found to be classical glycolytic enzymes α and γ enolase and glyceraldehyde 3-phosphate dehydrogenase (Adamus et al., 2011). Furthermore in a recent study patients with OCD

were tested for the presence ASOT and ABGA. Positivity for ABGA was observed in 19/96 (19.8%) patients and the majority of positive OCD sera (13/19) had antibodies against the enolase antigen (Nicholson et al., 2012).

Citrullination is the enzymatic conversion of arginine to citrulline *in situ* on proteins. This form of post-translational protein modification creates novel epitopes on common proteins, providing 'neoantigens' that are now known to be important in autoimmune diseases like rheumatoid arthritis (Fischer and Venables, 2012). In a perspective study by Lundberg et al., 2010 the hypothesis of molecular mimicry in the aetiology of RA has been explored with citrullinated enolase being the specific antigen involved. Both RA and periodontitis which is largely caused by *Porphyromonas gingivalis* infection have similar pathophysiology, characterized by inflammation. The citrullination of proteins by *P. gingivalis* and the subsequent generation of autoantigens that drive autoimmunity in RA might be representative of a causative link between these two diseases (Lundberg et al., 2008; Lundberg et al., 2010). Also numerous studies have demonstrated the presence of antibodies against citrullinated α -enolase in the serum samples from patients with RA (Kinloch et al., 2005; Montes et al., 2012).

1.5.3 Pyruvate kinase (PK)



Pyruvate kinase catalyses the transfer of a phosphate group from phosphoenolpyruvate (PEP) to ADP, yielding one molecule of pyruvate and one molecule of ATP, the final step of glycolysis. The PKM gene in humans is 32,315 kb long and consisting of 12 exons and 11 introns. Pyruvate kinase isoenzymes type M1 and M2 are splicing products of the PKM gene (exon 9 for M1-PK and exon 10 for M2-PK) (Noguchi et al., 1986; Dombrauckas et al., 2005).

Findings by Kostanyan and Nazaryan, 1996 demonstrate how pyruvate kinase induced glycolysis occurs in rat brains in response to oestradiol which may explain sex-specific phenomena such as chorea gravidarum and chorea whilst on the oral contraceptive pill (Kostanyan and Nazaryan, 1996). In addition monomeric pyruvate kinase acts as a T3/thyroid binding protein, with inhibition of the glycolytic enzymes activity occurring on binding (Kato et al., 1989, Ashizawa et al., 1991). This suggests that glycolytic enzymes are involved in the control of cellular metabolic effects that are induced by thyroid hormones. This could explain the association between hyperthyroidism and chorea (Cretel et al., 1998; Isaacs et al., 2005). Recent findings have shown that the expression and activity of PK is reduced in lymphocytes, polymorphic neutrophils (PMN) and leucocytes of normal pregnant women in comparison with those of non-pregnant women and pre-eclampsia patients which may explain why during pregnancy, many women experience remission of autoimmune diseases like multiple sclerosis and uveitis (Xu et al., 2010).

Pyruvate kinase M1/M2, the putative autoantigen in post-streptococcal disorders is expressed in differentiated tissues, such as lung, fat tissue, retina, pancreatic islets as well

as in all cells with a high rate of nucleic acid synthesis, which include all proliferating cells such as, embryonic cells, adult stem cells and tumour cells (Tsutsumi et al., 1988). This isoenzyme is known to be found mainly in the cytosol and to some extent in the nucleus. PK is associated with other glycolytic enzymes, i.e. glyceraldehyde 3-P dehydrogenase, hexokinase, phosphoglyceromutase, phosphoglycerate kinase, enolase and lactate dehydrogenase in a so-called glycolytic enzyme complex (Campanella et al., 2005). Immunoreactivity to PK in patients with exacerbated symptoms with a recent streptococcal infection was 7-fold higher compared to patients with exacerbated symptoms without evidence of a streptococcal infection (Church et al., 2002). Antibodies to PK were the most predominant amongst the glycolytic proteins identified as antigenic targets in the sera of patients with Sydenham's chorea (Church et al., 2002; Kansy et al., 2006). Additionally, the sera from patient with dementia and striatal hypermetabolism has been shown to selectively detected a 60 kDa protein (Léger et al., 2004).

1.6 CONCLUSION

As ABGA are strongly associated with recent streptococcal infection this group of disorders is a good model for the study of molecular mimicry and autoimmunity. Preliminary data suggest that ABGA have effects on neuronal function and may therefore be directly involved in the pathogenesis of basal ganglia dysfunction. As auto-antibody mediated diseases respond to immunomodulatory therapy, identifying and defining the pathogenesis of these disorders is important so that patients can be appropriately treated.

1.7 HYPOTHESIS

- Variation in the virulence factors (M protein and SAg) associated with GABHS might coexist between patients diagnosed with postulated post-streptococcal disorders and healthy controls.
- Inoculating mice with putative autoantigens (aldolase C, enolase and pyruvate kinase) in complete Freund's adjuvant will induce the development of ABGA in mice and result in stereotypical movements similar to those seen in human subjects with ABGA-associated neuropsychiatric disorders.
- Autoantibodies from patients with postulated post-streptococcal disorders including TS, EL, SC and dystonia will have functional effects *in vitro*, e.g. in the case of our identified putative autoantigens ABGA will affect neuronal function and survival.
- The transfer of these antibodies to animals will result in stereotypical movements similar to those seen in human subjects with ABGA-associated neuropsychiatric disorders.

1.8 AIMS

- To screen GABHS samples for *emm* type and SAg from patients diagnosed with postulated post-streptococcal disorders like TS, SC, EL and dystonia and healthy controls.
- To investigate the functional effects of ABGA from patients diagnosed with postulated post-streptococcal disorders like TS, SC, EL and dystonia in neuronal cell cultures.
- To produce an animal model of postulated post-streptococcal disorders by active immunization with putative autoantigens including aldolase C, enolase and pyruvate kinase.
- To produce an animal model of postulated post-streptococcal disorders by active immunization with group A-beta haemolytic streptococcal proteins.
- To produce an animal model of postulated post-streptococcal disorders by direct intra-cerebral infusion of autoantibodies from patients.

CHAPTER 2

GROUP A BETA-HAEMOLYTIC STREPTOCOCCUS (GABHS)

2.1 INTRODUCTION

2.1.1 GABHS Background

Lancefield group A streptococci consists of a single species, *S. pyogenes* which typically produces a large zone of beta-haemolysis when cultured on blood agar plates, and is therefore also called GABHS (Ryan and Ray, 2004). It is associated with a wide range of suppurative infections in the skin and respiratory tract, life threatening soft tissue infections and certain types of toxin-associated reactions (Hayes et al., 2001). Acute *S. pyogenes* infections may take the form of pharyngitis, scarlet fever, cellulitis or impetigo. Invasive infections can also result in necrotizing fasciitis myositis and streptococcal toxic shock syndrome (Hahn et al., 2005).

The spectrum of post-streptococcal disease of the CNS includes movement disorders (chorea, tics, dystonia and Parkinsonism), psychiatric disorders like OCD and ADHD and associated sleep disorders (Dale, 2005). The classical post-streptococcal disorders are known to be rheumatic fever and Sydenham's chorea which is a neurological manifestation of RF (Dale and Heyman, 2002; Dale, 2005).

GABHS express a number of cell surface components and extracellular products that play an important role in its pathogenesis and in the immune response of the host as discussed previously. Evidence suggests that GABHS contain antigens which are similar enough to host antigens and can stimulate B and T cells to respond to self. Of all the streptococcal antigens involved in molecular mimicry, the M protein has been best investigated and characterized (Bordeur, 2003). In addition streptococcal SAg cause non-specific activation of T-cells resulting in polyclonal T cell activation and massive cytokine release (Llewellyn and

Cohen, 2002). These extracellular pyogenic exotoxins are important as they cause direct tissue damage by initiation of apoptotic pathways or disruption of mammalian cell-walls (Watson, 1960; Hallas, 1985; Saouda et al., 2001). Although the pathophysiology of post-streptococcal disorders is not completely understood, antigenic mimicry is proposed to be the triggering cause of autoimmunity (Hahn et al., 2005, David Greenwood, 2007).

2.2 HYPOTHESIS AND AIMS

The major virulence factors determining the pathogenicity of streptococcal strains include M protein encoded by *emm* and *emm*-like (*emml*) genes and superantigens. The main hypothesis behind this study was that a differential pattern of *emm* type and superantigens might exist in the patients suffering from TS and dystonia as compared to neurological controls which might better explain the role of these two virulence factors in the disease pathogenesis. The aim of this study therefore was to investigate the *emm* types and superantigen profiles of GABHS isolates from patients diagnosed with suspected post-streptococcal movement disorders, mainly Tourette's syndrome, and neurological controls. I also wanted to see if there is a link between *emm* types and superantigens presented. In addition to the main aims above I wanted to select a sample that exhibited the most diverse range of SAg for creating an active immunization animal model (Chapter 3).

2.3 MATERIALS AND METHODS

All the work described in this chapter was performed at Great Ormond Street Hospital for Children, London, UK under direct supervision of Dr. Kathryn Harris (Clinical Scientist, Microbiology Unit, GOSH).

2.3.1 GABHS Samples

GABHS frozen on beads from 21 patients and 8 controls were assessed in this study. All samples were collected at Great Ormond Street Hospital, London during a cross-sectional and longitudinal study. The isolates were from patients diagnosed with TS with an age range of 11- 46 years. All controls samples came from healthy children with age ranging from 10- 16 years.

2.3.2 GABHS Culturing

GABHS samples frozen on beads were streaked onto solid agar plates supplemented with 5% horse blood. The plates were then incubated for 18-24 hours at 37°C aerobically with the addition of 5% CO₂. GABHS typically formed colonies 0.5mm in diameter surrounded by a zone of complete haemolysis. A swab of bacterial colonies was used to inoculate Todd-Hewitt broth for liquid cultures needed for preparation of bacterial homogenate and protein fractions.

2.3.3 Latex Agglutination Test

PROLEX™ streptococcal grouping latex kit (Pro-lab Diagnostics, UK) was performed for definitive serological identification of the cultured bacteria as GABHS. This streptococcal grouping method involves chemical extraction of group specific carbohydrate antigens using nitrous acid extraction reagents. The extraction reagents 1 and 2 provided in the kit contain a chemical substance to extract the streptococcal group specific antigens at room temperature. Extraction Reagent 3 contains a neutralizing solution. The neutralized extracts can be easily identified using latex particles sensitized with purified group specific rabbit immunoglobulins (IgG). These latex particles agglutinate strongly in the presence of homologous antigen and will not agglutinate when homologous antigen is absent.

1-4 beta-haemolytic colonies were selected using a disposable loop and suspended in one drop of Extraction Reagent 1 in a test tube. A drop of Extraction Reagent 2 was then added to the tube. The reaction was mixed by tapping for 5-10 s and approximately five drops of Extraction Reagent 3 was then added to the above reaction mix. One drop of group A streptococcal latex was dispensed onto a circle on the test card. A second streptococcal group specific latex was used as a negative control. Using a Pasteur pipette, one drop of colony extract was placed beside the group A streptococcal latex. The latex and the extract were then mixed with the sticks provided, using the complete area of the circle. The card was gently rocked allowing the mixture to flow slowly over the entire test ring area. At one minute, under normal lighting conditions, agglutination was observed.

2.3.4 *Emm* Typing

2.3.4.1 DNA isolation

GABHS samples from TS patients frozen on beads were streaked onto horse blood agar plates and incubated overnight at 37°C. A swab of each culture was put into a tube containing AE buffer (Qiagen, West Sussex, UK) and 0.1mm silica spheres (MP Biomedicals, UK). The samples were ribolysed at 6.5m/s for 40 seconds using the FastPrep®-24 Instrument (MP Biomedicals, UK) and centrifuged for 2 min at 17,500 x g. The supernatant was then transferred to clean Eppendorf tubes and the DNA was stored at 4°C until used.

2.3.4.2 Polymerase Chain Reaction (PCR)

Amplification of the emm gene was performed using the following primers:

Emm (Forward) - tatt (c/g) gcttagaaaattaa

Emm (Reverse) - gcaagttcttcagcttggtt

1µL (20pmol) of each primer was added to a PCR tube containing 1µL Q-BioTaq (5 units/µL, QBiogene Inc., UK), 5µL of 10x NH₄ buffer (Bioline Ltd., UK), 1.5 µL of 50 mM MgCl₂ and 0.4µL of 25 mM deoxynucleoside triphosphates (dNTPs). The final volume was adjusted to 50µL with PCR grade water. The reaction mixture was then amplified using the following PCR cycle in a T3000 thermocycler. The PCR products were analyzed on a 2% agarose gel with ethidium bromide and DNA hyper ladder II (Bioline Reagents Ltd., UK).

PCR Cycle

Step	Temperature (°C)	Time (in seconds)
1)Initialization	94	60
10 Cycles		
2)Denaturing	94	15
3)Annealing	46.5	30
4)Extension/Elongation	72	75
20 Cycles		
2)Denaturing	94	15
3)Annealing	46.5	30
4)Extension/Elongation	72	75
		With a 10 s increment for each of the subsequent 19 cycles.
5)Final Elongation	72	600 (10 minutes)
6)Final Storage	4	Short term storage

Table 2.1: PCR cycle used for emm typing of GABHS isolates.

2.3.4.4 Sequencing

The PCR products were purified using Illustra microspin S400H (GE Healthcare Life Sciences, Buckinghamshire, UK). The sequencing PCR mixture contained 2µL Big dye terminator (Applied Biosystems, California, USA), 1µL Sequencing buffer (Applied Biosystems, California, USA), 2.5µL (2pmol) of each primer, 1.5µL PCR grade water (Molzym, Germany) and 3µL of purified PCR product. The sequencing reactions were cleaned up by ethanol precipitation. 2µL EDTA (125mM) and 80µL of 96% ethanol were then added to the sequence reaction. The samples were incubated for 15 minutes at room temperature,

followed up by incubation on ice for 5 minutes. The mixture was then centrifuged for 15 minutes at 17,500 x g.

The supernatant was discarded and the pellet was washed with 200µL of 70% ethanol, spun for 5 min at 17,000 x g, the supernatant was discarded again and the pellet was air dried for 10 minutes. The pellet was resuspended in 10µL High Dye formamide (Applied Biosystems, California, USA), transferred to a 96-well plate and denatured for 5 min at 95°C in the PCR machine. The sequencing plate was then loaded into the genetic analyzer (ABI 3130, Applied Biosystems, California, USA).

Consensus sequences of the forward and reverse strands were generated with Seqman (Lasergene 8, DNASTar). Phylogenetic analysis was performed with MegAlign (Lasergene 8, DNASTar) using the ClustalW algorithm.

2.3.5 Superantigen Screening

2.3.5.1 PCR

DNA was extracted from the streptococcal cultures as described before. Individual PCR amplifications were undertaken to detect the genes for 13 known superantigens namely spe A, B, C, F, G, H, I, J, K, L, M, ssa and smeZ.

Superantigen primer sequences (Eurofins MWG Operon, Germany):

- 1) speA-up - 5'-ACTTAAGAACCAAGAGATGG-3'
speA-dn - 5'-CCTTATTCTTAGGTATGAAC-3'
- 2) speB-up - 5'-GGATCCCAACCAGTTGTTAAATCTCT-3'
speB-dn - 5'-AACGTTCTAAGGTTTGATGCCTACAA-3'
- 3) speC-up - 5'-AAGTGACTCTAAGAAAGACA-3'
speC-dn - 5'-TTGAGTATCAATGTTTAATG-3'

- 4) speF-up - 5'-CGAAATTAGAAAAGAGGAC-3'
speF-dn - 5'-GGCTGAGCAAAAGTGTGTG-3'

- 5) speG-up - 5'-CTGGATCCGATGAAAATTTAAAAGATTTAA-3'
speG-dn - 5'-AAGAATTCGGGGGAGAATAG-3'

- 6) speH-up - 5'-TTGGATCCAATTCTTATAATACAACC-3'
speH-dn - 5'-AAAAGCTTTTAGCTGATTGACAC-3'

- 7) speI-up - 5'-GGATCCATGAGTAGTGTGGGAGTTATTAA-3'
speI-dn - 5'-AAGCTTTTATTTATTAAATTTAACTAAG-3'

- 8) speJ-up - 5'-GGATCCGATAGTGAAAATATTAAAGCG-3'
speJ-dn - 5'-GAATTCGCTCCTATCTTATTTAGTCC-3'

- 9) speK-up - 5'-GCGGATCCGATACGTACAATACAAATG-3'
speK-dn - 5'-GCGAATTCAATAGCATTCAACCA-3'

- 10) speL-up - 5'-GCAGCCATATGGAAGAGACTATTCCTCTTAAGGATATATACTCTC-3'
speL-dn - 5'-GGGGATCCTTAATTTTCTTTGTTTGTGAATAAATAGAC-3'

- 11) speM-up - 5'-GCAGCCATATGTTTTTCAGATGCTGTGTTGG-3'
speM-dn - 5'-GGGGATCCTTAATTTTGTAGAAAAATCTTCGTTTAAGTA-3'

- 12) smeZ-up - 5'-TGGGATCCTTAGAAGTAGATAATA-3'
smeZ-dn - 5'-AAGAATTCTTAGGAGTCAATTTTC-3'

- 13) ssa-up - 5'-AGTCAGCCTGACCCTAC-3'
ssa-dn - 5'-TAAGGTGAACCTCTAT-3'

Each 50 µL reaction contained 20 pmol of each of the forward and reverse primers, PCR buffer (10 mM Tris/HCl, pH8.3; 50 mM KCl), 1.5 mM MgCl₂, 200 µM each dNTP, 2.5 U AmpliTaq Gold DNA polymerase and 10µL genomic DNA. The reaction mixture was then amplified using the below PCR cycle in a T3000 thermocycler. The PCR products were analyzed on a 2% agarose gel with 10 µL ethidium bromide and DNA hyper ladder II.

PCR cycle

Step	Temperature (°C)	Time (seconds)
1)Initializing	95	600 (10 minutes)
35 Cycles		
2)Denaturing	94	30
3)Annealing	50	30
4)Elongation	72	60
5)Final Elongation	72	120
6)Storage	4	Short term storage

Table 2.2: PCR cycle used for screening SAg in GABHS isolates.

2.4 RESULTS

2.4.1 *Emm* typing

GABHS DNA extracts from 21 patients and 8 child controls were *emm* typed using PCR followed by gene sequencing. In the patient cohort *emm* type 4 was the most predominant *emm* type and was found present in 8 out of 21 samples analysed (38%). This was followed by *emm*11 (19%), *emm*3 (14%) and *emm*1 (14%). Both *emm*1 and *emm*3 have been associated with invasive GABHS diseases. Another *emm* type exclusive to the isolates from the patient population was *emm*12. In the control samples *emm* types observed were *emm*4 (25%), *emm*78 (25%) and *emm*5, 6, 75 and 77 at low percentages. In both cohorts except for *emm*4 no other common *emm* types were observed

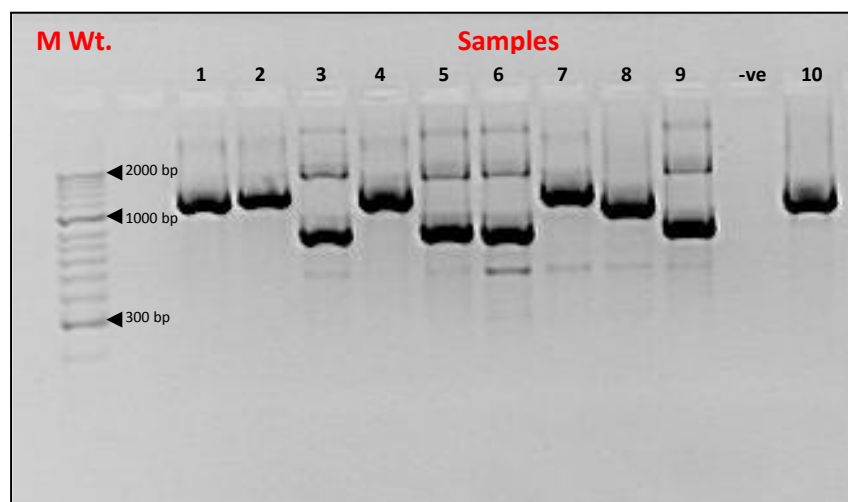


Figure 2.1: *Emm* typing PCR image. The above is an image of a PCR carried out as a first step for *emm* typing of GABHS isolates. The PCR product was then sequenced to assign an *emm* type. A negative control was also employed which came from Group B streptococcus isolate.

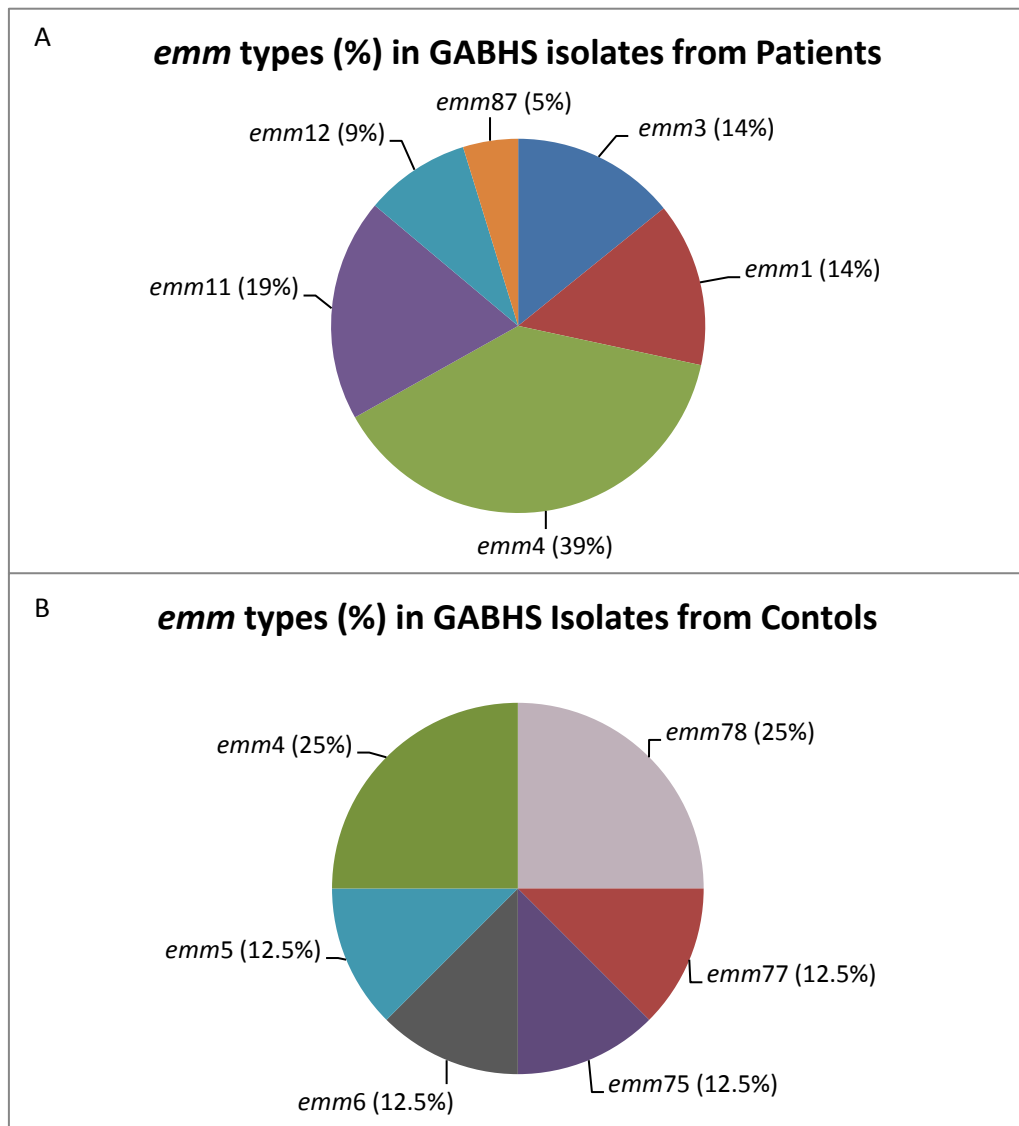
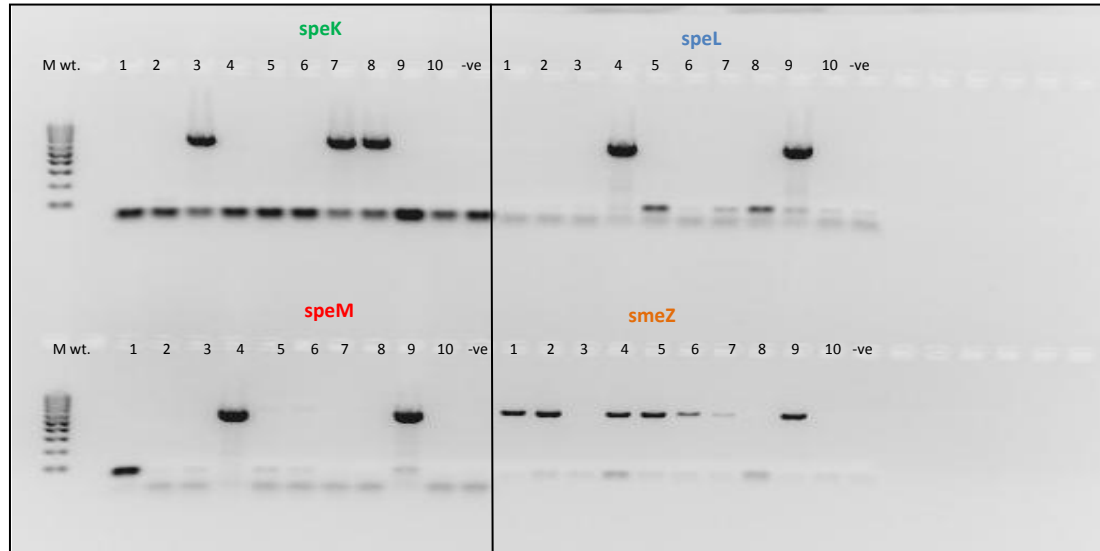


Figure 2.2: *Emm* types found in GABHS isolates from patients and controls. The pie chart above shows the *emm* types found in GABHS isolates from patients and controls as analysed through PCR and gene sequencing. The only common *emm* type found in both patients and control was *emm* type 4. **(A)** *Emm* types found in patient GABHS isolates consisted predominantly of *emm* type 4 followed by *emm*3 and *emm*11. **(B)** *Emm* types found in GABHS isolates from healthy controls presented with a high percentage of *emm*4 and *emm*78.

2.4.2 Superantigens

The same cohort of isolates was screened for the presence of superantigens speA, speB, speC, speF, speG, speH, speI, speJ, speK, speL, speM, ssa and smeZ using PCR.



Figures 2.3: PCR image for SAg screening (speK, L, M and smeZ) in GABHS isolates. PCR above shows the presence of superantigens speK, L, M and smeZ in some of the GABHS isolates analysed. speL and speM can be seen occurring in the same samples. The above PCR is representative of the PCRs carried out for SAg screening in the present study. The bands at the bottom of the PCR images show the formation of primer dimer.

SpeB was found in all 29 samples whereas speF was found in 28 out of the 29 samples with one control isolate not expressing it. SAg smeZ was present in 23 out of the 29 samples (79%) occurring in both cohorts. speL and speM were always co-detected which was expected, as the genes are in adjacent positions on the same bacteriophage (Smoot et al., 2002a). Both these SAg were observed in 5 isolates all belonging to the patient population. speA (14%), speJ and speK (19%) were found only in patients isolates. Only a single patient isolate showed the presence of speH and I. speC was seen in all but 5 isolates and was

present in both cohorts. To conclude speA, H, I, J, K, L and M were completely absent in the isolates from the control group.

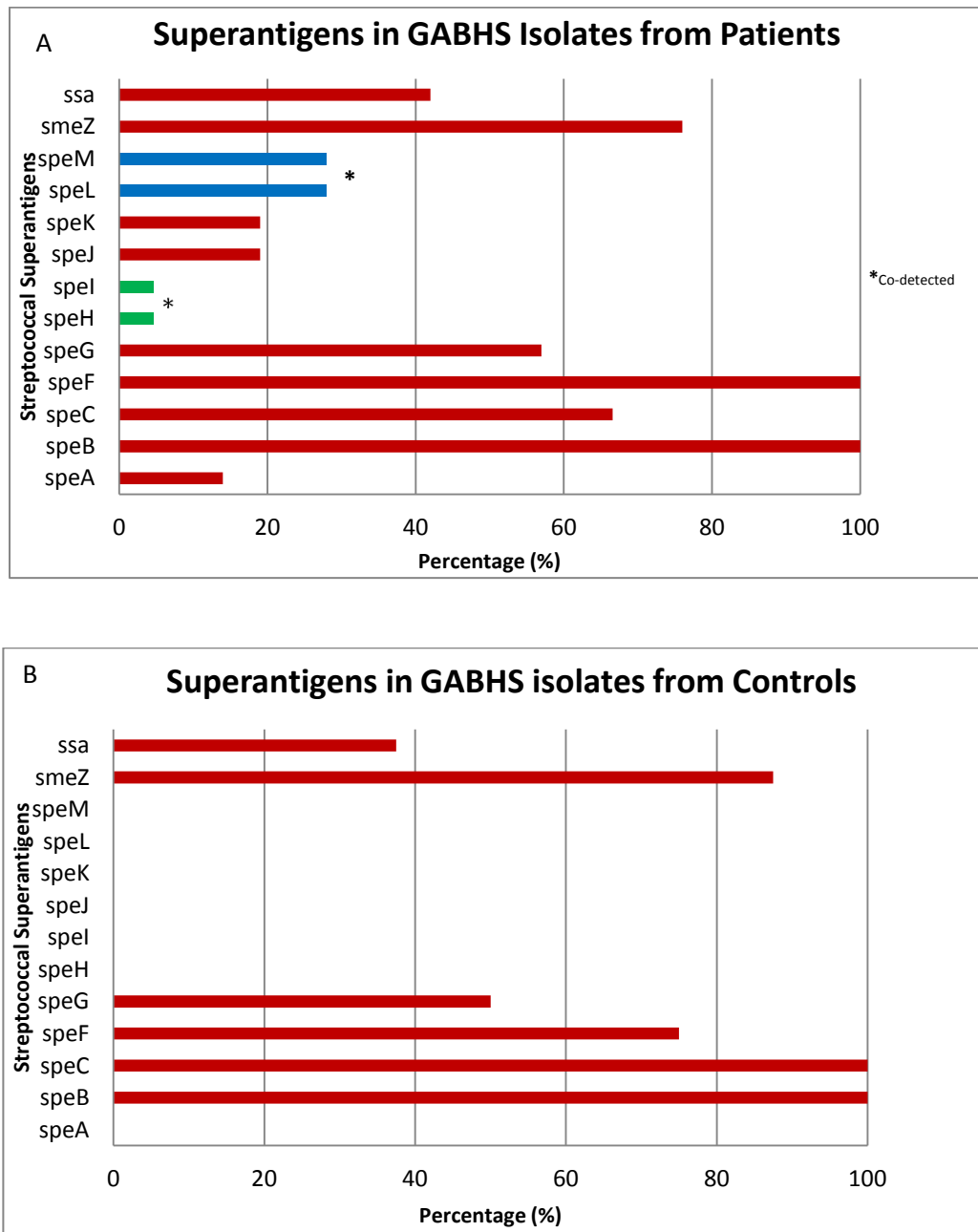


Figure 2.4: Superantigens present in GABHS isolates from patients and controls. The graphs above show the percentage of superantigens (speA, B, C, F, G, H, I, J, K, L, M, smeZ and ssa) that were detected in GABHS isolates from patients and neurological controls using PCR. **(A)** The percentage of each of the 13 known SA_g in 21 GABHS isolates from patients.

speB and F were seen in all samples; speH and I were always co-detected as were speL and M. (B) The percentage of each of the 13 known SAg in 8 GABHS isolates from controls. SAg speA, H, I, J, K, L and M were found to be completely absent from these GABHS samples; speB and speC were seen in all samples; speF was missing in 1 out of 8 samples.

2.4.3 Correlation: *emm* type and superantigens

In addition to the known linkage between the T serotype, serum opacity factor production, and the *emm* type, several studies also indicated correlations between *emm* types, disease manifestations, and other virulence factors, especially the superantigens. Therefore all the samples that were *emm* typed were also analysed for the presence of SAg to study the correlation between the two and the following was observed:

- SpeL and speM were always found in samples with *emm4*. Interestingly this was the case in GABSH isolates only from the patients as they were absent in *emm4* control isolates.
- Largest numbers of SAg were found to be present with *emm4* patient cases (B, C, F, G, J, L, M, smeZ and ssa). In contrast the control cases with the same *emm* type displayed a small number of SAg (B, C, F, smeZ, ssa). However, in both cohorts speG was found absent.
- SpeA was seen only with *emm1* and *emm3* isolates in the patients.
- SpeH and speI were the two superantigens present in *emm12* patient sample.
- All *emm3* samples did not have SAg smeZ.
- All *emm11* patient cases presented with the same SAg B, C, F, G, smeZ. Similarly, this was the case with *emm1* samples A, B, F, G, J, smeZ. In common to the above two *emm* type isolates was that they both lacked ssa.

PATIENTS	
Emmtypes	SAg
emm1	A, B, F, G, J, smeZ
emm3	A, B, F, G, K, ssa
emm4	B, C, F, L, M, smeZ, ssa
emm11	B, C, F, G, smeZ
emm12	B, F, G, H, I, smeZ
Others	B, C, F, G, smeZ, ssa
CONTROLS	
Emmtypes	SAg
emm4	B, C, F, smeZ, ssa
emm78	B, C, F, G, smeZ
Others	B, C, F, G, smeZ, ssa

Table 2.3: Observed co-relation between GABHS emm types and SAg. The table above shows the observed co-relation between emm types and SAg in both GABHS isolates from patients and controls. *speA* was only seen with emm1 and 3; *speL* and *M* only with emm4 but only in the isolates from patients and not controls; *speH* and *I* were only present in emm12 sample and *speJ* in emm1 and *speK* only in emm3. Most SAg that showed preference for a particular emm type like *speA*, *H*, *I*, *J*, *K*, *L* and *M* were absent in the control cohort.

2.5 DISCUSSION

GABHS is one of the most important pathogens encountered in clinical practice. Acute infections may take the form of pharyngitis, scarlet fever, impetigo, cellulitis, or erysipelas (Anand et al., 2012). Arrays of neurological and neuropsychiatric disorders have also been linked to GABHS like SC, TS, and OCD. GABHS express a number of cell surface components and extracellular products that play an important role in its pathogenesis. The pathogenicity of streptococcal strains is determined by major virulence factors like the M protein encoded by *emm* and *emm*-like (*emml*) genes and superantigens. Although the pathophysiology of post-streptococcal disorders is not completely understood, antigenic mimicry is proposed to be the triggering cause of autoimmunity (Hahn et al., 2005, David Greenwood, 2007, Greenwood et al., 2007).

In the present study GABHS samples were typed for the *emm* gene that encodes the M protein which is known to evoke cross-reactive antibodies, specific for human tissue including the brain. Also the aim was to determine if certain *emm* types were more prevalent in patients diagnosed with suspected post-streptococcal neuropsychiatric disorders as compared to neurological controls. In the 21 patient samples analyzed *emm4* (38.8%) seemed to be the most prevalent followed by *emm11* (19%) and *emm3* (14%). During 2002–2010, *emm4* was found to be the third most common *emm* type causing pharyngitis, and in 2007 and 2008, it was the most common *emm* type (Shia et al., 2011). In a study performed by Chiou et al., 2009 *emm4* (23.2%) again was found to be one of the prevalent *emm* type after *emm12* (50.4%) in patients with scarlet fever which is associated with immune complications including acute glomerulonephritis, rheumatic fever and erythema nodosum (Chiou et al., 2009).

In the current study *emm*11 was the second most prevalent *emm* type found in the isolates recovered from patients. Su and colleagues *emm* typed GABHS isolates from both invasive and non-invasive cases and observed that all other *emm* types were linked to both invasive and non-invasive infections except for type 11, which was uniquely associated only with invasive diseases (Su et al., 2009). *emm* type 3 found in 14% of the cases was the third most prevalent *emm* type found in patients followed by *emm*1. Both *emm*1 and *emm*3 have been established to be associated with the most severe GABHS diseases (Harari et al., 2009; Turner et al., 2012).

Different *emm* types tend to predominate in different age groups. In a study conducted by Meisal and colleagues in 2010 looking at *S. pyogenes* isolates causing severe infections in Norway between 2006 and 2007, *emm*12 was found to be common in 1- to 9-year-old patients, while *emm*4 was overrepresented in patients over 60 years of age (Meisel et al., 2010). These two *emm* types were found predominant as a cause of invasive infections in children (0 to 17 years) in Europe (Harari et al., 2009) and are also known to be common in noninvasive infections in children (Jaggi et al., 2005; Darenberg et al., 2007).

Predominant *emm* types in distinct geographic areas, often vary in frequency from year to year for reasons not fully understood. For instance the isolates from ARF patients in Hawaii presented with a completely different range of *emm* types (*emm* types 65/69, 71, 92, 93, 98, 103, and 122) than those in the United States (1, 3, 5, 6, 14, 18, 19, 24, 27, and 29) (Erdem et al., 2007).

Despite the considerable diversity of *emm* types of GABHS isolates, epidemiologic studies have found that relatively few *emm* types tend to predominate within a local population (Shulman et al., 2004; Shulman et al., 2009). These findings suggest that GABHS strains associated with a single disease may be quite different in geographical regions complicating

designing and customizing candidate vaccines to induce type-specific immunity. In addition, sizeable outbreaks can be caused by strains of a single *emm* type or of a small number of *emm* types. Overall, this combination of factors results in a complex epidemiologic situation for GABHS infections requiring a deeper understanding of geographic and temporal variation in *emm* types.

Next the superantigen profiles of the GABHS isolates from both patients and controls was analysed to determine whether there is any variation between the two cohorts. The results showed a range of superantigens to be only present in the patient isolates like *speA*, H, I, J, K, L and M which might be enhancing the immunopathogenicity of these isolates. Of these *speL* and M were discovered after the sequencing of genome of strain MGAS8232, a serotype M18 strain recovered from a patient with ARF (Smoot et al., 2002). Recombinant SPE-L (rSPE-L) and rSPE-M are seen to be highly mitogenic for human peripheral blood lymphocytes. Both SPE-L and SPE-M are shown to bind MHC class II antigens exclusively to the β -chain in a zinc-dependent binding mode and V β 1.1 TcR appeared to be the primary target of both the toxins and T cells carrying the V β 1.1 TcR being stimulated >80 times than with ConA (Proft et al., 2003). Other SAg uniquely seen in patient isolates was *speA* and GABHS that produce this exotoxin are known to be associated with severe infections characterized by rash, hypotension, multiorgan failure and a high mortality rate (Papageorgiou et al., 1999). It has also been linked to outbreaks of streptococcal toxic shock syndrome (STSS) in the United States and Europe. *SpeA* stimulates V beta 2.1, 12.2, 14.1, and 15.1-positive T cells, and the lymphokine production from the activated T cells is believed to result in the symptoms associated with STSS (Kline and Collins, 1997). *SpeJ* which was again found exclusively in the patient isolates has been found to be lethal in models of STSS. Purified recombinant *speJ* (rSPE J) expressed in *E. coli* stimulated the expansion of both human peripheral blood lymphocytes and rabbit splenocytes. The SAg

preferentially expanded human T cells displaying V β 2, -3, -12, -14, and -17 on their T-cell receptors (McCormick et al., 2001). SAg speJ was not found in all the samples in our study, this contrasts with the results of Proft and co-workers who observed a 100% prevalence suggesting this superantigen to be chromosomally encoded (Proft and Fraser, 2003; Proft et al., 2003).

Superantigen genes are thought not to be randomly distributed amongst GABHS isolates. Certain combinations of superantigen genes are found to be more common and the majority of *emm* types showed restricted superantigen profiles. Previous studies have also suggests that there might be a geographical variation in the association between superantigen gene possession and *emm* type (Ikebe et al., 2002). So in the present study I analysed any possible link between the two virulence factors and made a few observations like speL and M were found only in *emm* type 4 patient samples and not controls. This finding is consistent with those by Proft and colleagues who also found speL and speM in *emm*4, *emm*22, *emm*28 and 73% of *emm*89 isolates (Proft et al., 2003).

Next speG was found to be to be absent from *emm*4 isolates which has been in observed in some previous studies too (Murakami et al., 2002; Vlamincx et al., 2003; Common et al., 2008). This might suggest that *emm*4 isolates may contain an allele with mutations in the primer-binding sites (Commons et al., 2008). Also in accordance with all the previous studies it was found that smeZ was not present in *emm*3 isolates (Schmitz et al., 2003; Vlamincx et al., 2003; Commons et al., 2008). A disparity in binding sites in *emm*3 isolates would be constant with evidence that smeZ alleles are linked to *emm* type (Proft et al., 2000). Interestingly, both smeZ gene and *emm*3 isolates have been linked independently to STSS (Beres et al., 2002; Vlamincx et al., 2003). Previous studies (Descheemaeker et al., 2000; Vlamincx et al., 2003) have suggested that majority of *emm* type 1 isolates contain

speA, speG, speJ and smeZ but do not possess speC, ssa and speH which is once again consistent with findings reported here.

From the above observations it is clear that streptococcal SAg show *emm*-type-specific associations. The pattern seen in our study has been consistent with previous findings in the field making it possible for us to draw certain definitive conclusions about the correlation between the two virulence factors. However, the importance and relevance of these relationships needs further investigation by clarifying their role in the outcome of infection with GABHS.

To conclude *emm* types display variability in virulence gene profiles as well as differences in gender and age group preferences. The distribution of *emm* types in cases of invasive diseases also tends to vary over time and within different geographic regions. Same holds true for superantigens where no single unifying superantigen has been implicated. Accumulating evidence however is suggesting that these diseases are in response to one or many of a variety of superantigens in genetically susceptible individuals.

The patient and control streptococcal isolates were collected at Great Ormond Street Hospital for Children, London as part of a cross-sectional and longitudinal UK based study. The *emm* profile of GABHS can vary between different age groups, seasons and geographical locations. In the present study the patient and control cohorts were matched on the basis of season and geographical location but not for age which forms the biggest shortcoming of this study together with a small sample size. To take this study further I will be collaborating to work on a much larger cohort of TS and healthy children (approximately 100 per group). In addition, I aim to introduce an additional cohort consisting of siblings of TS patients as they share the same micro-environment. In the present work unfortunately, a large number of GABHS DNA extracts stored previously from both patients and controls

could not be analysed for *emm* and SAg as the DNA was not extractable. This could be due to sub-optimal long-term storage conditions or problems with the extraction itself. From the small number of samples analysed it appears that the SAg gene repertoire of the isolates correlate with the *emm* type in a complex pattern, preventing the formation of definite conclusions on the role of individual SAg in disease. The data presented should hence be taken forward analysing a large group of samples as it could be of value for preventive work, including ongoing attempts at creating vaccine prophylaxis against GA.

CHAPTER 3

ANIMAL MODELS

3.1 INTRODUCTION

In the present study it was attempted to create an animal model of postulated post-streptococcal neuropsychiatric movement disorders by active immunization with various putative antigens which are described below. Additionally a passive transfer model was created involving direct insertion of antibodies from patients in to the CNS.

3.1.1 Putative Antigen: GABHS Proteins

GABHS is a bacterium responsible for numerous human disorders because of its ability to induce immune-mediated sequelae. The M protein on the surface of GABHS has been identified as a possible antigen that ABGA may cross-react with (Dale et al., 2001). In support of this hypothesis, the M protein has been reported to induce antibodies that cross-react with α -helical human proteins. Furthermore, particular GABHS serotypes are associated with several post-streptococcal immune disorders, including SC (M types 5, 6, 19 and 24), ARF (M types 1, 3, 5, 6, 14, 18, 19, 24, 29) and glomerulonephritis (M types 1, 4, 12, 49, 55, 57, and 60) (Brandt et al., 2001; Church et al., 2002). In TS patients, increased titres of M12 and M19 serotypes have been observed; while antibodies from chorea patients have been shown to cross react with neuronal cells and M6 and M12 serotypes (Bronze et al., 1993; Müller et al., 2001). Similarly, antibodies from SC patients have been reported to cross-react with M types 5, 6 and 19 and several brain proteins ranging in size from 28kDa to 200 kDa. These neuronal proteins were not present in one particular anatomic region but the basal ganglia observed the most intense antibody interactions (Bronze and Dale, 1993).

Although streptococcal M protein is the most well characterized virulence factor a fairly complex picture is involved in host-pathogen crosstalk that involves a high number of proteins. Three categories of GABHS extracellular proteins have been recognized (Fischetti et al., 2000; Benfang et al., 2000). First category includes proteins that are actively secreted into the extracellular environment. Examples of these proteins include potent superantigens such as speA and C (Commons et al., 2008). The second category of proteins includes molecules with a secretion signal sequence and a conserved hexapeptide sequence (Leu-Pro-X-Th-Gly) that anchors the protein to the bacterial cell membrane (Fischetti et al., 2000). These cell surface-anchored proteins include known virulence factors such as M protein and C5a peptidase. In recent years, evidence has been presented a third category of GABHS extracellular proteins that lack apparent secretion signal sequences and the LPXTG membrane anchor motif. These proteins are found in the culture supernatants in high concentrations in the logarithmic phase of growth which may be related to passive release from the intracellular compartment via an unknown transport mechanism (Pancholi and Fischetti, 1992; Pancholi and Fischetti, 1998). Interestingly, two proteins in this class are glyceraldehyde-3-phosphate dehydrogenase and α -enolase both enzymes involved in the glycolytic pathway (Pancholi and Fischetti, 1993; Winram et al., 1996; Pancholi and Fischetti, 1998; Zhang et al., 2007).

3.1.2 Putative Antigen: Glycolytic Enzymes

Patients have been identified presenting with an encephalitic illness with a subsequent clinical course reminiscent of EL with ABGA and evidence of recent streptococcal infection (Dale et al., 2001; Dale et al., 2004a). ABGA have also been associated with other neuropsychiatric disorders including SC, PANDAS, TS and OCD. These autoantibodies are shown to recognise four main protein bands of 40kDa (aldolase C), 45kDa (neuron specific gamma enolase), 45 kDa (non-neuronal alpha enolase), 60 kDa (pyruvate kinase M1) and 98 kDa (neuron specific alpha/gamma dimer) that have been purified and successfully identified using 2-D gel electrophoresis and mass spectrometry (Dale et al., 2006). All the above antigens are glycolytic enzymes involved in energy homeostasis and as expected are found in the cytosol. However, these proteins are also shown to be located on the neuronal surface (Lim et al., 1983; Nakajima et al., 1994), where they appear to have “moonlighting” or alternative functions. These glycolytic enzymes/putative autoantigens have probable roles in energy metabolism, neurotransmission, cell health, maintenance of ion channels, and the control of cellular metabolism. All these roles are plausible and it is possible that autoantibodies could interfere with these roles either directly or indirectly (i.e. via a cell mediated response).

3.1.3 Animal Models

Autoimmunity has been postulated as the cause of at least some cases of a number of human diseases, including ankylosing spondylitis (Klebsiella), multiple sclerosis, Graves' disease, systemic lupus erythematosus (SLE), diabetes mellitus, rheumatoid arthritis, and atherosclerosis (Samarkos and Vaiopoulos, 2005). But the human disease with the best support for an autoimmune aetiology remains ARF (Bessen, 2001). Experimental allergic encephalomyelitis (EAE) provided some of the earliest evidence for the phenomenon of microbial-induced autoimmunity (Oldstone, 2000). Although research into autoimmune mechanisms is advancing at a rapid pace, the immune system response is still largely unexplained at the molecular level (Swedo and Grant, 2005).

Till date very few animal models have been set up to understand the aetiology and mechanisms of speculated post-streptococcal neuropsychiatric disorders the prototype being SC and TS. Briefly reviewed below are some of the animal models established so far to better understand the pathophysiology of these disorders and their link with GABHS.

3.1.2.1 Stereotypies models

TS and OCD are comorbid behavioral disorders, suggesting a shared but still unknown neuronal basis. Stereotypies and episodic utterances in animals have been proposed as a model of motor and vocal tics.

In an animal model developed by Taylor et al. (2002) sera from TS patients with high levels of anti-neural Abs, TS patients with low levels of autoantibodies and normal subjects were infused bilaterally into the ventrolateral striatum of male Sprague-Dawley rats. Rates of oral stereotypies (analogous to involuntary movements seen in TS) significantly increased in the rats infused with sera from the patients with high levels of autoantibodies. There was

also a marked increase in genital grooming in the rats infused with high-antibody TS sera, compared with the other groups. These results suggest that it is the presence of autoantibodies or other serum factor(s) lead to the observed stereotypies (Taylor et al., 2002). A similar study was carried out by Hallett et al. (2000) and their immunohistochemical analysis confirmed the presence of IgG that selectively bound to striatal neurons. These data support the hypothesis that binding of an anti-neuronal antibody from some children with TS induced striatal dysfunction and suggest a possible cause for the basal ganglia alterations observed in children with TS with the molecular mimicry hypothesis proposed for aetiology of a subgroup of TS cases (Hallett et al., 2000).

A study by Nordstrom and Burton (2002) used transgenic mice expressing a neuromodulating protein within a subset of dopamine D1-receptor expressing (D1+) neurons that are known to trigger glutamatergic excitation of orbitofrontal, sensorimotor, limbic and efferent striatal circuits. These circuits are thought to be affected in OCD and TS. These mice exhibited OCD-like behaviors including generalized behavioural perseveration and compulsion-like leaping and grooming-associated pulling and biting (Nordstrom and Burton, 2002). Additionally, the animals also exhibited comorbid TS-like behaviors, including juvenile-onset tics, increased tic number, complexity and flurries, increased tic severity in males, voluntary tic suppression. The importance of hormonal gender differences was highlighted in the study as this may be a factor sufficient to aggravate tic severity in human TS males compared to females (Nordstrom and Burton, 2002).

3.1.2.2 Active Immunization models

Only a limited number of animal models have been set up using GABHS to confirm its link with the onset of a spectrum of neuropsychiatric disorders in children.

The first animal model to test the hypothesis that an immune response to GABHS can result in behavioural abnormalities was carried out by Hoffman et al. (2004). Female SJL/J mice were immunized and boosted with a GABHS homogenate in Freund's adjuvant. Sera from GABHS immunized mice was then tested for immunoreactivity to mouse brain and a subset was found to be immunoreactive to several brain regions, including deep cerebellar nuclei (DCN), globus pallidus, and thalamus. GABHS-immunized mice also exhibited increased rearing behaviour in open-field and hole-board tests compared with controls and with GABHS-immunized mice lacking serum anti-DCN antibodies (Hoffman et al., 2004).

Another study was carried out by Yaddanapudi et al. (2009) in which they attempted to create a PANDAS mouse model following immunization with GABHS homogenate. Anti-CNS antibodies were seen directed against GABHS matrix (M) protein and cross-reacting with molecular targets complement C4 protein and alpha-2-macroglobulin in brain. They observed deficits in motor coordination, learning/memory and social interaction in PANDAS mice, replicating more complex aspects of human disease. Furthermore, they demonstrated that naive mice were transfused with IgG from PANDAS mice developed all the deficits listed above. This highlights that humoral immunity is necessary and sufficient to induce the syndrome. Their work provides an insight into PANDAS pathogenesis and may lead to new strategies for identification and treatment of children at risk for autoimmune brain disorders (Yaddanapudi et al., 2009).

A model of SC has been recently described by Brimberg et al. (2012) using male Lewis rats which when exposed to GABHS antigen exhibited motor symptoms (impaired food manipulation and beam walking) and compulsive behaviour (increased induced-grooming). Consistent with the known pathophysiology of these neuropsychiatric disorders antibody deposition was found in the striatum, thalamus, and frontal cortex. Concomitant alterations in dopamine and glutamate levels were seen in the cortex and basal ganglia. Furthermore, the motor symptoms were found to improve by the D2 blocker haloperidol and the selective serotonin reuptake inhibitor paroxetine, respectively, these are the drugs used to treat motor symptoms and compulsions in streptococcal-related neuropsychiatric disorders (Brimberg et al., 2012). These results were consistent with the hypothesis that an immune response to GABHS can cause motor and behavioural disturbances and suggest that anti-GABHS antibodies cross-reactive with brain components may play a role in their pathophysiology.

3.2 HYPOTHESIS AND AIMS

Inoculating mice with GABHS proteins or recombinant proteins (putative autoantigens) in complete Freund's adjuvant will induce the development of anti-neuronal antibodies in mice and result in stereotypical movements similar to those seen in human subjects with ABGA-associated neuropsychiatric disorders.

Induction of autoantibodies from patients with postulated post-streptococcal neuropsychiatric disorders like EL in mice should result in stereotypical movements similar to those seen in human subjects. If enolase is indeed a target autoantigen in this group of disorders then injecting mice with monoclonal antibody (mAb) against neuron specific enolase (NSE) should also result in stereotypical movements similar to those seen in human subjects.

The main aim of this study was develop a mouse model that resembled the behavioural and locomotor deficits associated with TS and other proposed streptococcal-related neuropsychiatric disorders by:

(1) Active immunization with a cocktail of the putative autoantigens aldolase C, α -enolase, γ -enolase and pyruvate kinase. **(2)** Active immunization with GABHS protein fractions (soluble + insoluble). **(3)** Intra-cerebral induction of autoantibodies purified from patients and controls and mAb against enolase.

3.3 MATERIALS AND METHODS

3.3.1 GABHS Protein Fractions

GABHS isolate used for protein extraction belonged to a TS patient and was *emm* type 4 presenting with a range of superantigens *speB*, C, G, F, L, M, *smeZ* and *ssa*. The isolate frozen on beads was streaked onto a solid agar plate supplemented with 5% horse blood. The plate was then incubated for 18-24 hours at 37°C aerobically with the addition of 5% CO₂. GABHS typically formed colonies 0.5mm in diameter surrounded by a zone of complete haemolysis. A swab of bacterial colonies was used to inoculate Todd-Hewitt broth (Sigma-Aldrich, UK) for liquid cultures needed for preparation of insoluble and soluble protein fractions.

GABHS colonies were extracted from the Todd-Hewitt broth using successive rounds of centrifugation to form pellets in the bottom of 25mL falcon tubes. The bacterial pellet was re-suspended using 1mL of 20mM Tris-HCL buffer (pH7.5) with 20 μ L of protease inhibitor cocktail (Sigma-Aldrich, UK). To remove the soluble proteins, 0.1mm beads were added to each re-suspension which was then put in a bead beater for 90 s. The suspension was then centrifuged at 10,000 x g for 30 min and the supernatant collected. This supernatant formed the soluble (membrane) fraction and was stored at -80°C until required. The

remaining bacterial pellet were re-suspended in 1mL of 20mM Tris-HCL buffer (pH 7.5) and centrifuged at 10,000 x g for 15 min. The supernatant was removed and discarded. The pellet was then re-suspended in 8M urea and rocked for 1 h followed by centrifugation at 10,000 x g for 30 min. The supernatant was collected which formed the insoluble protein fraction and was stored at -80°C until required.

3.3.2 Production of Recombinant Proteins

3.3.2.1 pQE30 Vector

The pQE-30 expression vector from the The QIAexpress® System (Qiagen, UK) used in our study as it provides a high-level expression of 6xHis-tagged proteins in *E. coli*. It is based on the T5 promoter transcription–translation system. The pQE plasmids belong to the pDS family (Bujard et al., 1987) and offer some important features like:

- Optimized promoter–operator element consisting of phage T5 promoter and two *lac* operator sequences.
- Synthetic ribosomal binding site, RBSII, for high translation rates
- 6xHis-tag coding sequence either 5' or 3' to the cloning region
- Nickel-nitrilotriacetic acid (Ni-NTA) metal-affinity chromatograph matrices for biomolecules which have been tagged with 6 consecutive histidine residues
- Multiple cloning site and translational stop codons in all reading frames for convenient preparation of expression constructs
- Two strong transcriptional terminators: *tO* from phage lambda (Schwarz et al. 1987), and T1 from the *rnB* operon of *E. coli*, to prevent read-through transcription and ensure stability of the expression construct.

3.3.2.2 Transformation using XL1-Blue Competent cells

XL1-blue competent cells (Agilent Technologies, UK) were used for transformation. These are ready to use bacterial cells (*E.coli*) chemically treated to allow incorporation of foreign DNA/plasmid. This strain allows blue-white color screening for recombinant plasmids and is an excellent host strain for routine cloning applications using plasmid or lambda vectors.

Two 14-mL BD Falcon polypropylene round-bottom tubes were pre-chilled on ice. (One tube was for the experimental transformation and one tube was for the pUC18 control). The SOC medium was pre-heated to 42°C. XL1-Blue competent cells were then thawed on ice and then gently mixed and aliquoted into each of the two pre-chilled tubes (50µL of cells per tube). A volume of 0.85µL of β-mercaptoethanol provided with the kit was added to each of the above cell aliquots. The contents of the tubes were gently swirled and then incubated on ice for 10 min, with gentle swirling every 2 min. Following the incubation 0.1–50 ng of the pQE-30 vector (1µL) was added to one of the aliquots of cells and to the other 1µL of the pUC18 control DNA was added. The tubes were then swirled gently again and incubated on ice for 30 min. The tubes were then heat-pulsed in a 42°C water bath for 45 seconds and then incubated on ice for 2 min. The duration of the heat pulse is critical for maximum efficiency. A volume of 0.45mL of preheated SOC medium was then added to the tubes which were then incubated at 37°C for 1 h with shaking at 650 x g. A volume of 100µL of the transformation mixture was then plated onto LB agar plates containing ampicillin which were then incubated overnight for at least 17 h at 37°C. The following day 2X5mL of LB broth was inoculated using a single colony from the above plate. This broth was then incubated for approximately 17 h at 37°C with shaking at 650 x g. DNA extraction was then performed using the QIAprep Spin Miniprep Kit (Qiagen, UK).

3.3.2.3 Vector Digestion with Restriction Enzymes

Alpha Enolase and Aldolase C: BamHI and HindIII

PK: KpnI and BamHI

Gamma Enolase: KpnI and HindIII

The restriction enzymes (New England Biolabs Inc., UK) were chosen for each of the four recombinant proteins using the NEBcutter V2.0 (New England Biolabs Inc., UK). The pQE30 vector DNA was then digested using the restriction enzymes HindIII and BamHI for α -enolase and aldolase-C, KpnI and BamHI for pyruvate kinase and KpnI and HindIII for γ -enolase as described below.

A 20 μ L reaction mixture was prepared with 800 ng of the pQE30 vector DNA + 2 μ L of 10X BSA + 20 units (\sim 1 μ L) of the first restriction enzyme + 2 μ L of 10X NE Buffer 1/2/3 (as appropriate to that restriction enzyme). This reaction was then incubated at 37°C for 1 h in a heat block following which the second restriction enzyme was added together with sodium chloride. The reaction mix was incubated for a further 1h at 37°C in a heat block. The restriction reaction was stopped by storing at -20°C. The following day the reaction was run on a 0.7% agarose gel containing SYBR® Safe (Invitrogen, UK). The DNA fragment was excised from the agarose gel with a clean, sharp scalpel using a trans-illuminator and gel extraction was performed as described below.

3.3.2.4 Gel Extraction

Gel extraction was carried out using the QIAQuick Gel Extraction Kit (Qiagen, UK). The gel slice was weighed in an eppendorf tube and to 1 volume of agarose gel (100mg \sim 100 μ L) 3 volumes of Buffer QG was added. The maximum amount of gel slice per QIAquick column

was 400 mg. This was then incubated at 50°C for 10 min (or until the gel slice has completely dissolved). To help dissolve the gel, the tube was vortexed every 2–3 min during the incubation. After the gel slice has dissolved completely, it was checked that the colour of the mixture is yellow if the colour of the mixture was orange or violet, 10µL of 3 M sodium acetate, pH5.0, was added and mixed. Next 1 gel volume of isopropanol was added to the sample. This step increases the yield of DNA fragments <500 bp and >4 kb. The QIAquick spin column was then placed in a provided 2mL collection tube. To bind DNA, the sample was simply applied to the QIAquick column, and centrifuged for 1 min. Once the flow-through was discarded 0.5mL of Buffer QG was applied to the column which was then centrifuged for a further 1 min. Following this the column was washed with 0.75mL of Buffer PE and centrifuged again for 1 min. Once the flow-through was discarded QIAquick column was centrifuged for an additional minute at 17,000 x g. The DNA was then eluted in a clean eppendorf tube by addition of 50µL of Buffer EB (10 mM Tris·Cl, pH8.5) or dH₂O (pH 7.0–8.5) to the centre of the QIAquick membrane and centrifugation for 1 min. The concentration was measured using Nanodrops and the DNA sample was then stored at -20°C until used for ligation.

3.3.2.5 Clones

Clones for the four recombinant proteins were obtained from Source Bioscience Geneservice Cambridge, UK. The IMAGE IDs of the clones are: Homo sapiens Alpha Enolase-**2906988**/LLCM43-J13(M13R) cDNA clone MGC: 2414, Homo sapiens Aldolase C-**2987869**/LLCM76-D14(M13R) cDNA clone MGC: 1449, Homo sapiens Gamma Enolase-**3629603**/IRAU21-F2(M13F) cDNA clone MGC: 2309 and Homo sapiens Pyruvate Kinase-**3859987**/IRQL4-E12(M13R) cDNA clone MGC:9369.

The four cDNA clones were streaked on to LB agar plates containing ampicillin (100µg/mL) and incubated at 37°C for 16-18 h. Using a colony from each plate 5mL of LB broth containing ampicillin was inoculated and incubated at 37°C for 16-18 h with vigorous shaking at 600 x g. DNA was extracted using the QIAprep Spin Miniprep Kit (Qiagen, UK) as described below.

3.3.2.6 DNA extraction

The QIAprep Spin Mini Prep kit (Qiagen, UK) was used for DNA extraction. The pelleted bacterial cells were resuspended in 250µL Buffer P1 and transferred to a microcentrifuge tube. 250µL Buffer P2 was then added and mixed thoroughly by inverting the tube 4–6 times followed by addition of 350µL Buffer N3 and which was mixed immediately and thoroughly by inverting the tube 4–6 times again. The mixture was centrifuged for 10 min at 17,900 x g in a table-top microcentrifuge. The supernatants were then applied to the QIAprep spin column by decanting or pipetting. This was again centrifuge for 30–60s and the flow-through discarded. The QIAprep spin column was then washed with 0.5mL Buffer PB and centrifuged for 30–60s. After discarding the flow-through the QIAprep spin column was washed with 0.75mL of Buffer PE and centrifuged for 30–60s. The column was then again centrifuged for an additional 1 min to remove residual wash buffer. To elute the DNA, 50µL Buffer EB (10 mM Tris-Cl, pH8.5) or distilled water was added to the center of the QIAprep column placed in a clean 1.5mL microcentrifuge tube. The DNA concentration was measured using Nanodrops.

3.3.2.7 PCR

Designing the primers

Knowledge of the sequence to be amplified and the vector into which the resulting cDNA is to be ligated allowed us to design primers that amplify the desired sequence and introduce appropriate restriction sites into the cDNA. By incorporating a different restriction site at each end of the amplified sequence cloned products could be directionally inserted into an appropriate vector. This was achieved by extending the 5' sequence of the oligonucleotide primers used in the PCR to introduce the appropriate restriction enzyme recognition sequence. Amplification of the DNA from the four clones was performed using the following primers:

GGATCC- BamHI

AAGCTT- HindIII

GGTACC- KpnI

Glycolytic Enzyme	Primer Sequence
Ald C (Forward)	ATGTAG GGATCC CCTCACTCGTACCCAGCCCT
Ald C (Reverse)	GATCT AAGCTT AGTAGGCATGGTTGGCAATG
α -Eno (Forward)	CATAC GGATCC TCTATTCTCAAGATCCATGCAG
α -Eno (Reverse)	AGTCA AAGCTT TACTTGGCCAAGGGGTTTCTG
γ -Eno (Forward)	CAGTA GGTACC CCATAGAGAAGATCTGGGC
γ -Eno (Reverse)	TGCTCA AAGCTT TCACAGCACAAGTGGGATTAC
PK (Forward)	CATAC GGATCC TCGAAGCCCCATAGTGAAGC
PK (Reverse)	TCTAT GGTACC CACGGCACAGGAACAACAC

Table 3.1: Primer sequences for glycolytic enzymes. Forward and reverse primer sequences with the appropriate restriction site for glycolytic enzymes ald C, α -enolase, γ -enolase and pyruvate kinase.

5 μ L of 10x NH₄ buffer, 2.5 μ L of 50 mM MgCl₂, 5 μ L of dNTP, 1 μ L (20pmol) of each primer, was added to 1 μ L Q-BioTaq (5 units/ μ L, QBiogene Inc., UK) and 5 μ L of 1:100 DNA sample was added to a PCR tube and the final volume was adjusted to 50 μ L with PCR grade water.

The reaction mixture was then amplified using the following PCR cycle in a thermocycler. The PCR products were analyzed on a 2% agarose gel with ethidium bromide and DNA hyper ladder II (Bioline Reagents Ltd., UK).

PCR Cycle

Step	Temperature (°C)	Time
1)Initialization	94	10 min
25 Cycles		
2)Denaturing	94	30 s
3)Annealing	46.5	30 s
4)Extension/Elongation	72	120 s
5)Final Elongation	72	10 min
6)Final Storage	4	Short term storage

Table 3.2: PCR cycle used for amplification of glycolytic enzymes aldolase C, α -enolase, γ -enolase and pyruvate kinase.

3.3.2.8 PCR Purification

DNA fragments were purified using the QIAquick PCR Purification Kit (Qiagen, UK). 5 volumes of Buffer PB was added to 1 volume of the PCR sample and mixed. The sample was then applied to the QIAquick column placed in a provided 2mL collection tube and centrifuged for 30–60 s to bind the DNA. After the flow-through was discarded the QIAquick column was placed back into the same tube and washed with 0.75mL of Buffer PE followed by centrifugation at 10,000 x g for 30–60 s. Again the flow-through was discarded and the column was centrifuged for an additional 1 min at maximum speed. After centrifugation the QIAquick column was placed in a clean 1.5mL microcentrifuge tube and DNA was

eluted by adding 50µL Buffer EB (10 mM Tris·Cl, pH8.5) or dH₂O to the centre of the QIAquick followed by centrifugation for 1 min. The DNA concentration was then measured using Nanodrops.

3.3.2.9 Restriction Reaction

Digestion of the DNA from the four clones was carried out with the chosen restriction enzymes as described above for the pQE30 vector.

Alpha Enolase and Aldolase C: BamHI and HindIII

Gamma Enolase: KpnI and HindIII

PK: KpnI and BamHI

3.3.2.10 Ligations

A ligation reaction consisting of 200ng of pQE30 expression vector + 30ng of insert (both digested by restriction enzymes) was prepared. To the above 1µL of T4 DNA ligase (New England Biolabs Inc., UK) and 2µL of 10X Buffer for T4 DNA ligase were added and the final volume was adjusted to 20µL with dH₂O. T4 DNA ligase catalyzes the joining of two strands of DNA between the 5'-phosphate and the 3'-hydroxyl groups of adjacent nucleotides in either a cohesive-ended or blunt-ended configuration. The ligation reaction was then put in the PCR machine at 16°C for approximately 50 h. Following the incubation transformation was carried out using XL1-Blue competent cells (as described previously). The transformed cells were plated onto LB agar plates containing ampicillin and incubated for 17 h at 37°C. Two colonies from each vector + insert plate were then used to inoculate 5mL LB broth which was then incubated for a further 16 h at 37°C with shaking at 550 x g. Plasmid DNA purification was carried out as per the instructions in the Qiagen plasmid maxi prep kit

(Qiagen, UK). The samples were then sequence verified at The Genome Centre, William Harvey Research Institute.

3.3.2.11 Induction of proteins using Isopropyl β -D-1-thiogalactopyranoside (IPTG)

Once sequence verified protein expression was induced using Isopropyl β -D-1-thiogalactopyranoside (IPTG) (Sigma-Aldrich, UK). IPTG is used in cloning procedures requiring induction of β -galactosidase activity. 10mL of culture medium containing ampicillin (100 μ g/mL) was inoculated in a 50mL flask and the cultures were grown overnight at 37°C. The following day 100mL of prewarmed media (with antibiotics) was inoculated with 5mL of the overnight cultures and incubated at 37°C with vigorous shaking until an optical density (OD₆₀₀) of 0.6 was obtained which took approximately 30-60 min. 1mL of sample for each protein was taken immediately before induction which served as a non-induced control. Expression was induced in the rest of the culture by addition of 1 mM IPTG. The cultures were incubated for an additional 4–5 h after which a second 1mL sample was collected which was the induced control sample. Both the non-induced and induced samples were pelleted and re-suspended in SDS-PAGE buffer and stored at -20°C until analysis by gel electrophoresis. The remaining cells were then harvested by centrifugation at 4000 x g for 20 min. The solubility of the target proteins was then determined by re-suspending the cell pellet in 5mL of lysis buffer for native purification. The samples were then snap frozen in dry ice and thawed in cold water followed by sonication 6 x 10s with 10s pauses at 200–300 W. The lysate was kept on ice at all times. Centrifugation was then carried out at 10,000 x g at 4°C for 20–30 min. The supernatant was decanted (soluble protein) and saved on ice. The remaining pellet was then resuspended in 5mL lysis buffer. This formed the suspension of the insoluble matter (insoluble protein). Protein solubility was then determined by SDS-PAGE analysis and staining with Coomassie stain and

immunodetection using anti-His antibody as defined below. Once the solubility of the target proteins was confirmed appropriate protocol was followed for native or denaturing conditions (described).

3.3.2.12 Anti-His Immunodetection

The proteins were separated by SDS-PAGE using a 10% Tris-HCl ready gel (Bio-Rad, UK) and the membrane was stained with 0.5% Ponceau S + 0.1% acetic acid. Once the protein bands were visible the membrane was washed 3X10 min with TBS buffer following incubation for 1 h in blocking buffer 3% BSA in TBS at RT. The membrane washed 2X10 min with TBS buffer before incubation with 1:1000 anti-His antibody (Qiagen, UK) prepared in blocking buffer at RT for 1h. Again the membrane was washed 2X10 min with TBS-tween. Incubation with horseradish peroxidase (HP) conjugated anti-mouse IgG (Sigma-Aldrich, UK) secondary antibody solution prepared in nonfat dried milk in TBS was carried out for 1h at RT. The membrane was then washed 4X10 min in TBS-Tween buffer. Chemiluminescent detection reaction was then performed and expose to X-ray film according to the manufacturer's recommendations.

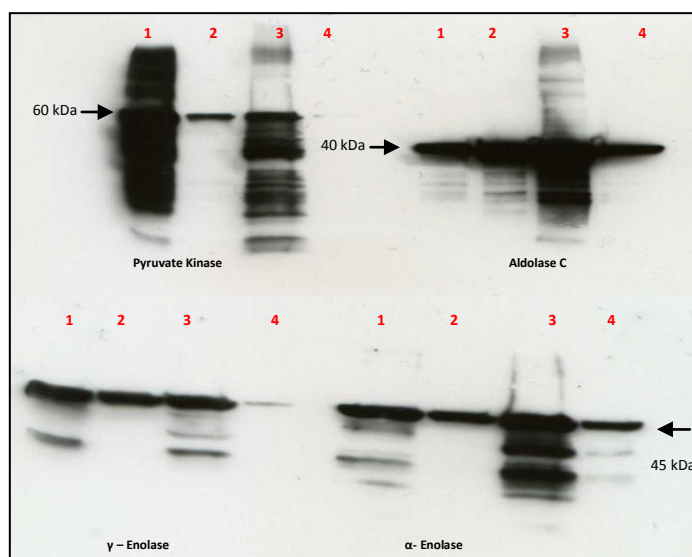


Figure 3.1: Western blot confirming the expression of recombinant proteins. Western blot confirming the expression of the four recombinant proteins (aldolase C, α -enolase, γ -enolase and pyruvate kinase) detected by anti-his antibody. Lane 1: soluble fraction; lane 2: insoluble fraction; lane 3: induced sample (IPTG induction); lane 4: non-induced sample.

3.3.2.13 Protein Purification

Native Conditions (Soluble Fraction)

The pellet derived from 50mL cell culture volume was re-suspended in 6.3mL of lysis buffer (50 mM NaH_2PO_4 , 300 mM NaCl, 10 mM imidazole, pH8.0). 700 μL lysozyme stock solution (10 mg/mL) and 3 Units/mL culture volume Benzonase® Nuclease (Novagen, 25 U/ μL) was then added to the above. The mixture was then incubated on ice for 15–30 min followed by centrifugation at 12,000 x g for 15–30 min at 4°C. The supernatant was collected out of which 20 μL of the cleared lysate was saved for SDS-PAGE analysis. The Ni-NTA spin column was equilibrated with 600 μL the lysis buffer and centrifuged for 2 min at 890 x g. Upto 600 μL of the cleared lysate containing the 6xHis-tagged protein was loaded onto the pre-equilibrated Ni-NTA spin column. Centrifugation for 5 min at 270 x g was then carried out

and the flow-through collected. Again a small sample of the flow-through was saved for analysis by SDS-PAGE to check for binding efficiency. The Ni-NTA spin column was then washed twice with 600µL of wash buffer (50 mM NaH₂PO₄, 300 mM NaCl, 20 mM imidazole, pH8.0) and centrifuged for 2 min at 890 x g. Again the flow-through (wash fractions) was saved for analysis later by SDS-PAGE to check the stringency of the wash conditions. The proteins were then eluted twice with 300µL of elution buffer (50 mM NaH₂PO₄, 300 mM NaCl, 500 mM imidazole, pH8.0) by centrifugation for 2 min at 890 x g.

Denaturing Conditions (Insoluble Fraction)

The pellet was thawed and re-suspended in 700µL of Buffer A (7 M urea; 0.1 M NaH₂PO₄; 0.01 M Tris·Cl; pH8.0) to which 3 Units/mL culture volume Benzonase® Nuclease (Sigma-Aldrich, UK) was also added. The cells were incubated with agitation for 15 min at RT. The solution should become translucent when lysis is complete. The lysate was then centrifuged at 12,000 x g for 15–30 min at RT (20–25°C) to pellet the cellular debris and the supernatant was collected. 20µL of the cleared lysate was stored for SDS-PAGE analysis. The Ni-NTA spin column was then equilibrated with 600µL of Buffer A and centrifuged for 2 min at 890 x g. The cleared lysate supernatant containing the 6xHis-tagged protein was then loaded onto the pre-equilibrated Ni-NTA spin column. Again centrifugation was carried out for 5 min at 270 x g, and the flow-through collected. A small sample of the flow-through was saved for analysis by SDS-PAGE to check binding. The Ni-NTA spin column was then washed with 600µL Buffer B (8M urea; 0.1M NaH₂PO₄; 0.01M Tris·Cl; pH6.3) and centrifuged for 2 min at 890 x g. Once again the flow-through was saved for analysis by SDS-PAGE to check the stringency of the wash conditions. The protein was then eluted twice with 200µL Buffer C (8 M urea; 0.1M NaH₂PO₄; 0.01M Tris·Cl; pH4.5) by centrifugation for 2 min at 890 x g.

3.3.3 Active Immunization Animal Model

3.3.3.1 Strains and Treatment Groups

Female Biozzi ABH and SJL mice purchased from Harlan, UK were used for these studies. The mice were approximately 10 weeks old and were housed for 2 weeks before the start of the experiment. Animals were maintained and studies were carried out in accordance with the Animals (Scientific Procedures) Act 1986. A total number of 8 animals per treatment group were used for both studies: This number was selected as previous studies attempting to find autoantigens have been successful (Amor S et al., 1994).

Group 1: CFA only; **Group 2:** Recombinant proteins (200µg of each of the four recombinant proteins: aldolase C, α -enolase, γ -enolase and pyruvate kinase); **Group 3:** GABHS soluble + insoluble proteins (400µg per fraction)

3.3.3.2 Active Immunization

The immunization strategy was based on the protocol used to induce myelin targeted autoimmunity (Al-Izki et al., 2012).

20mL syringes were used to make up the solution (i.e. multiples of 20mL). Firstly a stock solution was prepared (stock A), consisting of 4mL incomplete Freund Adjuvant (Difco, Becton Dickinson, UK), 16mg *Mycobacterium tuberculosis* H37Ra and 2mg *M. butyricum* (Difco, Becton Dickinson, UK), in a 5mL Bijou. Complete adjuvant: Freund's complete adjuvant was prepared by adding 11.5mL incomplete Freund's adjuvant to 1mL stock A that was vortex-mixed before use.

The plunger from a 20mL syringe was removed and the barrel was plugged with a stopper cap. For the recombinant protein cocktail 200µg of each of the four proteins was added to

5mL sterile PBS. Similarly for the GABHS protein cocktail 400µg of both insoluble and soluble protein fractions were added to 5mL sterile PBS. This was mixed and then 5mL of Complete Freund's adjuvant was added to both mixes. The syringes were sealed with parafilm and vortexed. A retort stand, boss and clamp was used to hold the 20mL syringes in place with the water level reaching the level of the adjuvant (containing a drop of detergent) in a waterbath sonicator (Branson Ultrasonicator, Sigma, UK) and sonicated for 10 min to thicken the mixture. The adjuvant was vortexed and placed on ice to cool. A 1mL syringe (Becton Dickinson, UK) was inserted into the 20mL syringe and the adjuvant was pumped using the 1mL syringe until it had thickened sufficiently that the solution did not disperse when a drop was added to water. The plunger was inserted into the 20mL syringe and the syringe was tapped on the bench such that the content moved towards the plunger and then the syringe cap was removed. A long (6cm) large bore needle was fixed to the syringe and inserted into 1mL syringes with plungers pulled out to the 1mL mark. The syringe was filled to 1mL and the barrel of the 1mL syringe was wiped with tissue paper to remove any excess adjuvant. A 16mm 25g needle (Becton Dickinson, UK) was fixed to the 1mL syringe. With the tip of the needle cover on the bench, the syringe was pushed very firmly onto the needle.

Mice were held at the nape of the neck between thumb and forefinger. The tail was held with the right hand with thumb and forefinger (tips facing the head) and the mouse was placed on the top of a wire mouse cage. The skin of the dorsal surface of the flank was lifted with thumb and forefinger (left hand) and the needle was inserted (facing towards the head) subcutaneously into the mouse. A volume of 0.10mL of adjuvant was injected into the right flank and another 0.10mL was injected into the left flank. This was day 0. The procedure was repeated at day 7 and day 25. Injections at the latter time points were made more posterior to the original injections.

3.3.3.3 Assessment

The mice were weighed before the start of the experiment and then every day after day 12. All animals were subjected to a baseline assessment using the open field activity chamber and rotarod for 300 s each. These tests were then repeated 5 days after every immunization i.e., day 5, 12 and 30.

Open field activity chamber: The open field can be used to measure hyperactivity, exploratory activity, stereotyped rotation and locomotor impairment. It is also commonly used as a measurement of anxiety-related behavior (Belzung and Griebel, 2001). The test was carried out in an open field Plexiglas chamber equipped with multiple photocell receptors and emitters. The activity of the animal was then measured via a grid of invisible infrared light beams. The body of the mouse, when placed within the monitor, caused some of these beams to be broken, thus revealing its position in the (X-Y) plane. The analyzer collected the beam status information from the activity monitor and the computer software calculated multiple variables such as total distance travelled, horizontal activity, vertical activity, and movement time over a period of 5 min.

Rotarod: This test was used to assess the animal's motor coordination and balance. The mouse was placed on a cylinder that can rotate at constant or accelerating speeds for a period of 300 s. Control and experimental subjects were compared for their ability to remain on the rod either by latency to drop off or the final speed achieved by the accelerating rod before they fall.

3.3.5 Intra-Cerebral Animal Model

3.3.5.1 Sera Samples

Antibodies (IgG) were purified from sera of a patient diagnosed (1) EL (2) dystonia (3) healthy control. The EL patient was a female in her mid-twenties. The purified antibodies from both patients and controls were then injected intra-cerebrally in Biozzi ABH mice as described later.

3.3.5.2 Immunoglobulin G (IgG) Purification

Collection tubes were prepared by adding 60-200µL of neutralisation buffer (1M Tris-HCl pH9.0) per mL of fraction to be collected. The pump was then filled with 10mL of binding buffer. HiTrap Protein G column (GE Healthcare, UK) was then attached to the pump tubing. Introduction of air into the column should be avoided. The snap-off end at the column outlet was then removed and the column was washed with 10mL of binding buffer (0.02M sodium phosphate pH7.0) at 1mL/min. The filtered sera sample was then applied to the column using the pump at 1-3mL/min. This was followed by washing the column with 5-10mL of binding buffer until no material appears in the effluent. The antibodies were eluted with 2-5mL of elution buffer (0.1M citric acid pH4.5-5.5) and collected into tubes containing the neutralisation buffer as described in step 1. After elution the column was re-equilibrated with 5-10mL of binding buffer. Before storage the column was washed with 20% ethanol to prevent microbial growth and stored at 4- 8°C.

3.3.5.3 Animals and Treatment Groups

Inbred female Biozzi ABH mice were used for this study .The mice were approximately 9-10 weeks old before the start of the experiment. Animals were maintained and studies were carried out in accordance with the Animals (Scientific Procedures) Act 1986. A total number of three animals per treatment group were used:

GROUP	TREATMENT
1	Purified EL IgG (0.5 mg/mL)
2	Purified EL IgG (0.05 mg/mL)
3	Purified dystonia IgG (1 mg/mL)
4	Purified dystonia IgG (0.1 mg/mL)
5	mAb against NSE (1 mg/mL)
6	mAb against NSE (0.1 mg/mL)
7	Purified healthy controls IgG (1 mg/mL)
8	Purified healthy controls IgG (0.1 mg/mL)

Table 3.3: Intra-cerebral animal model treatment groups. The table above summaries the various antibodies used for intra-cerebral injection in mice.3 animals were used per treatment group.

3.3.5.4 Intra-cerebral injection

Inbred Biozzi ABH mice were anaesthetised using isoflurane which is an inhalant anaesthetic. Surgical levels of anaesthesia of this general anaesthetic were maintained in oxygen/nitrous oxide. Once under general anaesthesia the mice were injected with 30µL of purified IgG from patient sera using 0.5mL Myjector U-100 insulin syringe with 29g x 0.5 needle (Terumo, USA). The injection site was aimed at the forebrain (sight for the basal

ganglia) halfway along the sagittal suture before it meets the bregma suture. Although the injection site was aimed at the right frontal cortex but because of the volume injected this can spread around the brain and reach the spinal cord (Baker et al., 1992). Three mice per experimental group were employed for this study to see that the effects were reproducible. A short video of each animal was made once injected with the purified IgG. The animals were killed by dislocation of the neck as described in the Code of Practice for the Humane Killing of Animals under Schedule 1 to the Animals (Scientific Procedures) Act 1986.

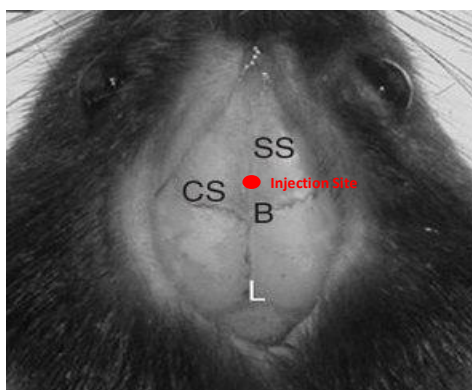


Figure 3.2: Injection site of autoantibodies in the mouse brain. The injection site was aimed at the forebrain to target the basal ganglia approximately halfway along the sagittal suture (SS) before it meets the bregma suture (B). The other two sutures shown above are cranial suture (CS) and lambda suture (L).

3.3.6 Enzyme Linked Immunosorbent Assay (ELISA)

Indirect ELISA was performed to analyse the reactivity of the serum from the test and CFA only animals against the four recombinant proteins and GABHS protein fractions. 10µg of the individual proteins and protein cocktail prepared in carbonate coating buffer were coated onto 96 well flat bottom culture plates. The plate was incubated overnight at 4°C. The following day the plate was blocked with 1% BSA prepared in PBS for 1 h at 37°C. The

plate was then washed 4-5 times with PBS+0.1% tween following which the primary antibody (serum from animals immunized with recombinant proteins/GABHS proteins and CFA) was added. The plate was incubated for a further 1.5 h at 37°C. Once again the plate was washed 5 times with PBS + 0.1% tween before the addition of HP conjugated anti-mouse secondary antibody prepared at 1:1000 dilution. After 1 h of incubation at 37°C the washes were repeated and 100µL of TMB substrate was added to each well. After the reaction turned blue it was stopped by addition of 50µL of hydrochloric acid (HCl). The plate was then read at 450 nm in an ELISA plate reader using the KC-4 programme.

3.3.7 Haematoxylin and Eosin (H&E) Staining

Tissue Fixation and Embedding:

The tissue collected from the animals was fixed using 4% paraformaldehyde for 48-72 h. Once fixed the tissue was transferred into plastic cassettes for overnight processing in Tissue-Tek VIP (Sakura). The following day the tissue was embedded in paraffin wax before being sectioned (5µm) using a microtome onto polysine slides (VWR, UK). The slides were allowed to dry overnight and the sections were stained as described below:

Rehydration

Xylene (2X)	5 min
100% EtOH	3 min
100% EtOH	3 min
90% EtOH	3 min
70% EtOH	3 min
dH ₂ O	5 min

Staining

Haemotoxylin 1-5 min

Rinse dH₂O

Acid Alcohol 1-6 quick dips.

(0.1% HCL in 50% EtOH)

Tap Water 5 min

Eosin 3 min

Rinse in a few quick changes of dH₂O

Dehydration

70% EtOH 1 min

90% EtOH 2 min

100% EtOH 2 min

100% EtOH 2 min

Xylene (2X) 3 min

The slides were cover slipped using DEPEX mounting medium and viewed under the light microscope.

3.4 ACTIVE IMMUNIZATION MODEL RESULTS

3.4.1 Weight

All animals were weighed before the start of the study and then every day after day 10. No significant drop was observed in the weight of the mice during the duration of the experiment. A slight drop was observed after every immunization but that is a normal phenomenon owing to the stress caused by immunization.

3.4.2 Rotorod

All animals were assessed for motor coordination and balance using a rotorod. The test was carried out before the start of the experiment i.e., pre-immunization and then 5 days after every immunization. No significant difference was observed between the mice immunized with GABHS proteins or recombinant proteins as compared to CFA only controls.

3.4.3 Open Field Activity Chamber

3.4.3.1 ABH Animal Model

Behavioural and locomotor assessment of ABH mice was done using an open field activity chamber at various time points: pre immunization and then 5 days after every immunization carried out at day 5, 12 and 30. The data was collected in the dark and each animal was allowed to acclimatize to the chamber before the test was started. The results show a decrease in total distance travelled by animals immunized with recombinant protein after the third immunization as compared to pre-immunization ($p=0.05$). However, no significant difference was observed when they were compared to CFA immunized control animals. In contrast, ABH mice immunized with GABHS fractions showed a significant decrease in average total distance travelled when compared to CFA controls ($p=0.02$) and baseline measurement ($p=0.005$).

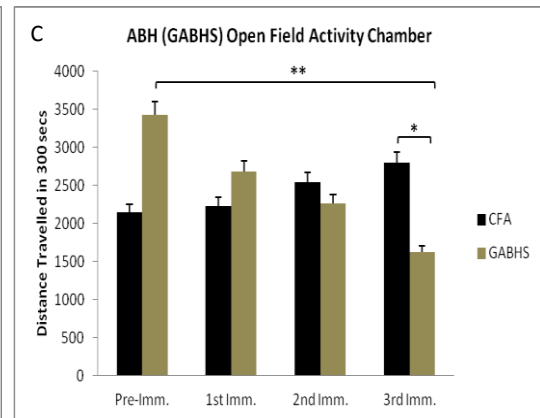
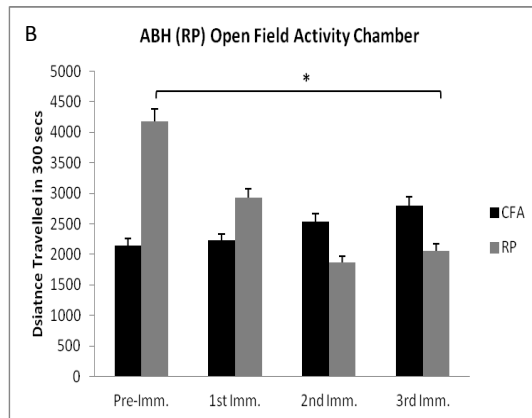
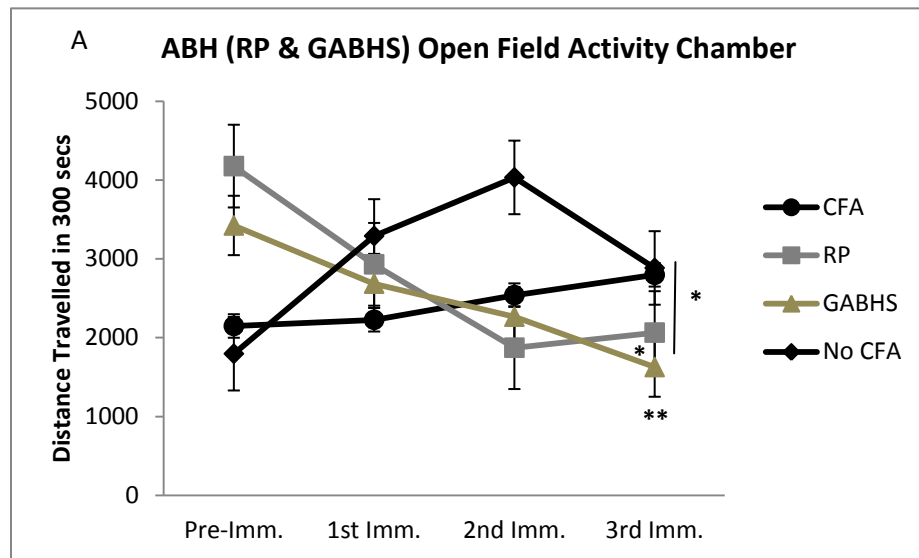


Figure 3.3: Average distance travelled by ABH mice immunized with GABHS proteins, RP and CFA using an open field activity chamber. Graphs A, B and C above depicts the average distance travelled by ABH mice immunized with GABHS protein fractions, recombinant proteins (ald C, α and γ -enolase and PK) and CFA in 300s using an open field activity chamber. **(A)** A combined graph of distance travelled by ABH mice in the test and control group. A two tailed T-test confirmed a significant decrease in distance travelled by test mice after the third immunization as compared to pre-immunization (recombinant proteins $p < 0.05$; GABHS proteins $p < 0.005$). Also a significant difference was observed in the distance travelled between the GABHS and CFA only mice ($p < 0.02$) which was not seen in

recombinant protein mice. **(B)** Graph comparing the average distance travelled by recombinant protein mice to CFA only control mice. No significant difference in the distance travelled was observed between the test and control group. However, a significant decrease was observed between the pre-immunization and after the third immunization in the test group ($p<0.05$). **(C)** Graph comparing the average distance travelled by GABHS protein mice to CFA only control mice. A significant decrease in average distance travelled was observed in mice immunized with GABHS proteins as compared to controls after the third immunization ($p<0.02$). Similarly a significant difference in motor activity was seen in test mice between pre-immunization and after the third immunization ($p<0.005$).

3.4.3.2 SJL Animal Model

Behavioural and locomotor assessment of ABH mice was done using an open field activity chamber at various time points: pre immunization, and 5 days after every immunization carried out at day 5, 12 and 30. The data was collected in the dark and each animal was allowed to acclimatize to the chamber before the test was started. The results show a decrease in total distance travelled by animals immunized with recombinant protein after the third immunization as compared to pre-immunization ($p<0.05$). However, no significant difference was observed when they were compared to CFA immunized control animals. In contrast, ABH mice immunized with GABHS fractions showed a significant decrease in average total distance travelled when compared to CFA controls ($p<0.02$) and baseline measurement ($p<0.005$).

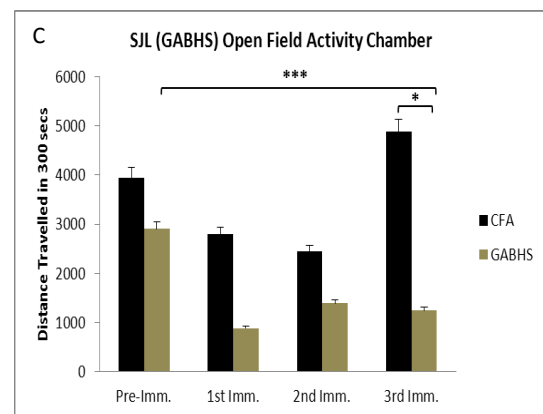
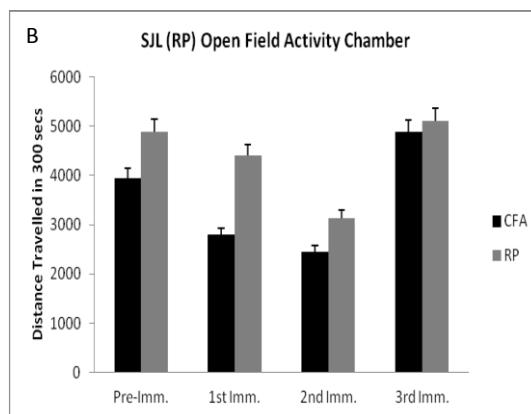
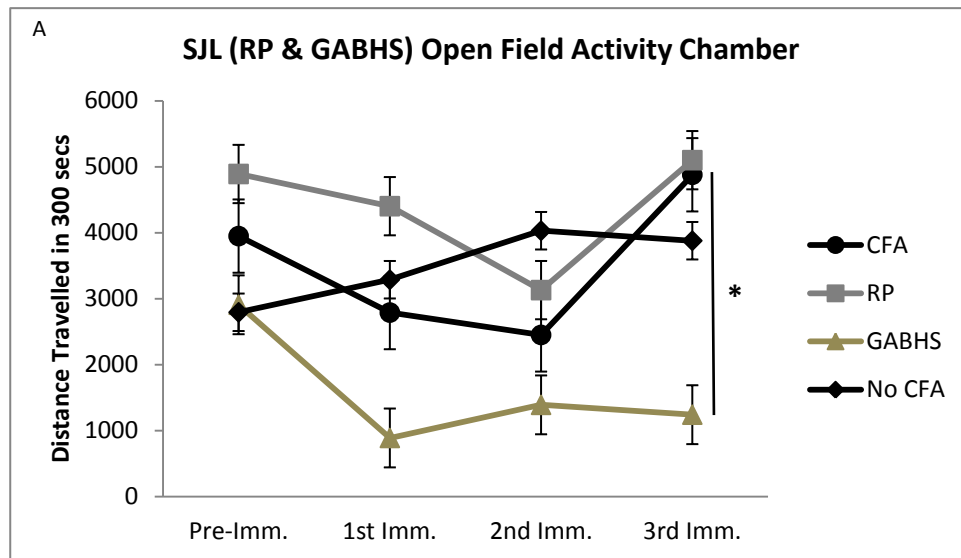


Figure 3.4: Average distance travelled by ABH mice immunized with GABHS proteins, RP and CFA using an open field activity chamber. The graphs A, B and C above depict the average distance travelled by SJL mice immunized CFA, GABHS protein fractions and recombinant proteins (ald C, α and γ -enolase and PK) in 300s using an open field activity chamber. **(A)** A combined graph of distance travelled by SJL mice in the test and control group. A two tailed T-test confirmed a significant decrease in distance travelled by test mice after the third immunization as compared to pre-immunization only in the GABHS mice ($p < 0.02$). Also a significant difference was observed in the distance travelled between the GABHS compared to CFA only mice ($p < 0.001$) which was not seen in recombinant protein

mice. **(B)** Graph comparing the average distance travelled by recombinant protein mice to CFA only control mice. No significant difference in motor was observed between the test and controls group or between the various immunization time points in the test group. **(C)** Graph comparing the average distance travelled by GABHS immunized mice to CFA only controls. A significant decrease in average was observed in mice immunized with GABHS proteins as compared to CFA controls after the third immunization ($p<0.02$). A significant difference was also observed in motor activity in test mice between the pre-immunization and after the third immunization recording ($p<0.001$).

3.4.4 Serum reactivity to antigens

ELISA was performed to test the serum reactivity of animals immunized with various antigens compared to CFA only controls. Sera of animals immunized with the a cocktail of the four recombinant proteins ald C, α - eno, γ - eno and PK was tested for reactivity against the four individual proteins to see if any particular protein elicited a higher immune response. Results showed a significantly higher serum reactivity of the test group as compared to CFA controls to all four proteins and the cocktail ($p=0.01-0.001$). Furthermore, serum reactivity to γ -enolase ($p<0.001$) and PK ($p=0.01$) was observed to be higher than to ald C and α -enolase in both strains of mice.

Similar ELISA was performed for GABHS protein fraction comparing reactivity of the test animals with the controls. Once again a highly significant difference was observed between the serum reactivity of the animals immunized with GABHS soluble fraction ($p=0.0002$) and insoluble fraction ($p=0.001$) to the CFA controls. However, no significant difference was observed between the reactivity to insoluble and soluble protein fractions.

3.4.4.1 Recombinant Proteins

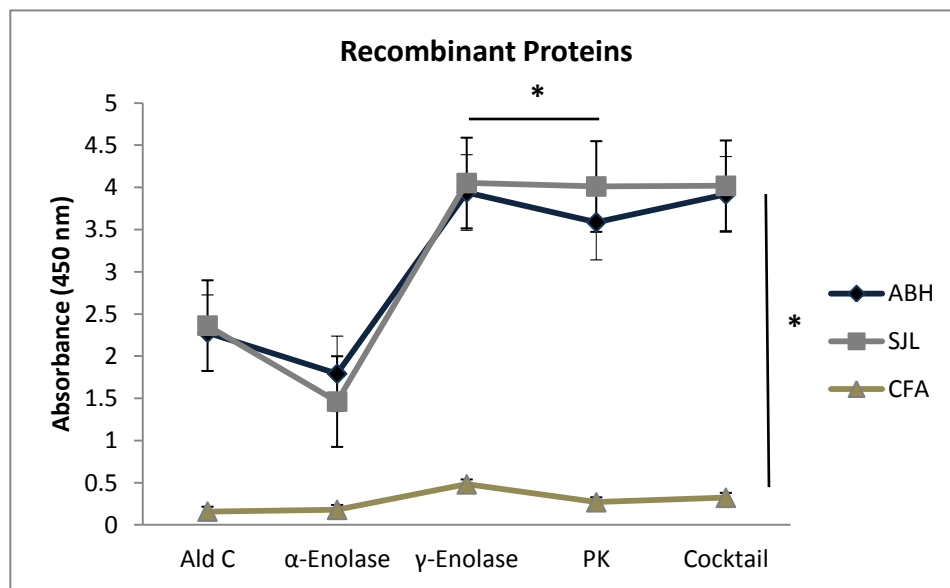


Figure 3.5: ABH and SJL sera reactivity to recombinant proteins (autoantigens). The line graph above shows the reactivity of sera from ABH and SJL animals immunized with recombinant proteins (ald C, α and γ-enolase and PK) compared to CFA only control animals to individual proteins and a cocktail of all four recombinant proteins. A two-tailed t-test revealed a highly significant difference in reactivity of the sera from immunized animals as compared to CFA controls in both mouse species (p values ranging from 0.01 to 0.0001). Additionally higher sera reactivity was seen against γ-enolase ($p < 0.001$) and PK ($p < 0.01$) compared to ald C and α-enolase in both ABH and SJLs.

3.4.4.2 GABHS Proteins

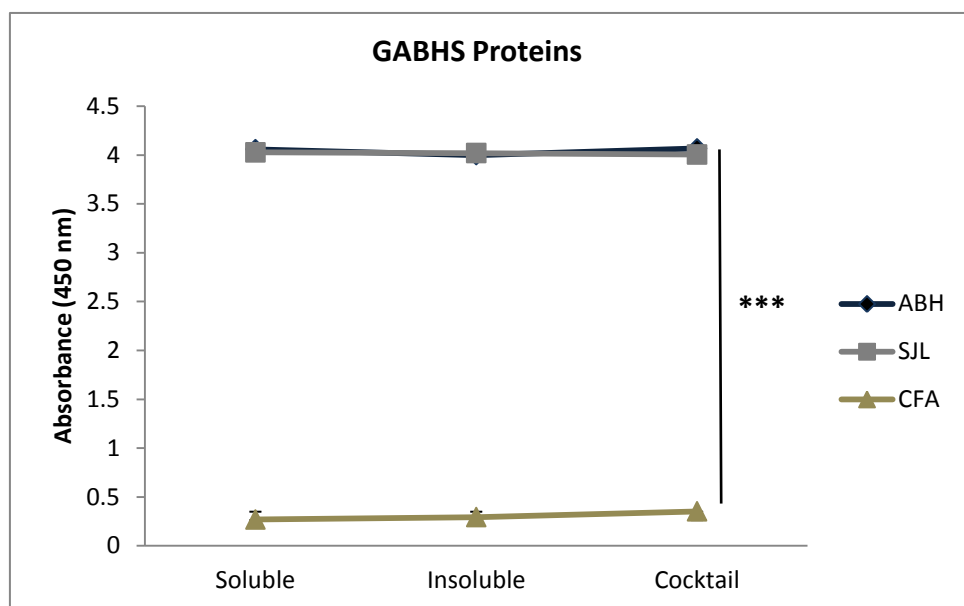


Figure 3.6: ABH and SJL sera reactivity to GABHS protein fractions. The graph above shows the reactivity of sera from ABH and SJL animals immunized with GABHS protein fractions as compared to CFA only control animals to individual GABHS fractions (insoluble and soluble) and a cocktail. A two tailed t-test revealed a highly significant difference in reactivity of the sera from immunized animals as compared to CFA controls in both mouse species ($p < 0.001$ to 0.0001). However, no difference was observed between the reactivity to insoluble and soluble fraction.

3.4.5 Assessment of Inflammation (H&E)

H&E staining was carried out on both the heart and brain tissue from ABH and SJL mice immunized with GABHS proteins, recombinant proteins and CFA controls to examine them for any signs of inflammation. The brain tissue from ABH animals immunized with GABHS protein fractions showed massive leukocyte infiltration in the midbrain region. The cells are suspected to be neutrophils because if you look at higher power you can observe multilobal nuclei. However additional staining is required to confirm this. Similarly what appears to lymphocyte infiltration was seen in brain tissue of ABH mouse immunized with the four recombinant proteins (aldC, α -enolase, γ -enolase and PK). No infiltration was observed in the tissues recovered from SJL animals or heart tissue from ABH.

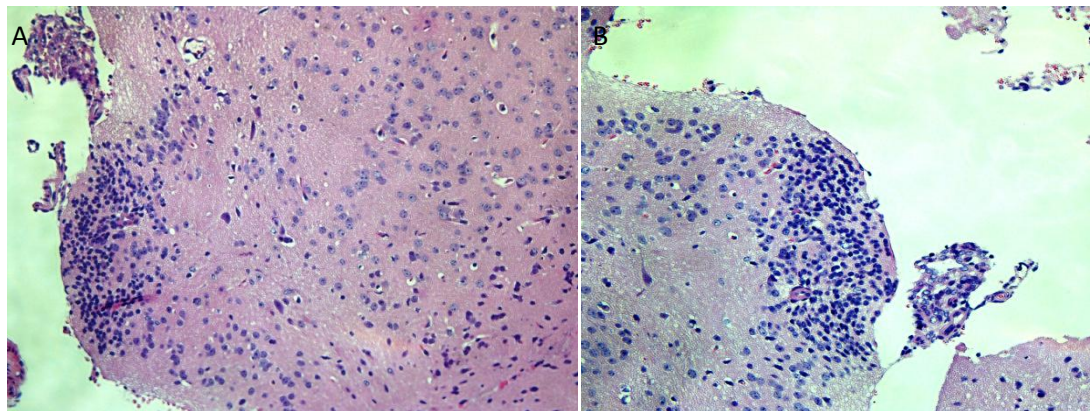


Figure 3.7: H&E stained brain sections from ABH mice immunized with GABHS proteins and recombinant proteins. 20X Images A & B above show H&E stained brain sections from ABH mice immunized with GABHS proteins and recombinant proteins (ald C, α and γ -enolase and PK). Infiltration was observed in brain sections from both groups and the deep staining cells might possibly be lymphocytes. **(A)** Midbrain section from ABH mouse immunized with GABHS protein fractions. **(B)** Midbrain section from ABH mice immunized with recombinant proteins.

3.5 Intra-Cerebral Animal Model Results

The injection of the antibodies was done blinded to the nature of the antibody used. Any animals with signs of brain damage as a result of the injection were excluded from the study.

3.5.1 EL IgG

Female Biozzi ABH mice were injected intra-cerebrally with a volume of 30 μ L of purified IgG from EL patient sera at concentration of 0.5 and 0.05 mg/mL. Three mice were used per experimental group. Induction of antibodies at a concentration of 0.5 mg/mL produced pronounced signs like tremors and tail rigidity both of which are reminiscent of EL symptoms. These signs occurred with a rapid onset following administration, but were transient as many of these resolved within 1 h of treatment. However, to a lesser degree most of these stereotypical movements were still observed after induction of antibodies at a tenfold dilution (0.05 mg/kg). Injection of PBS and non CNS specific IgG failed to induce any signs in the animals.

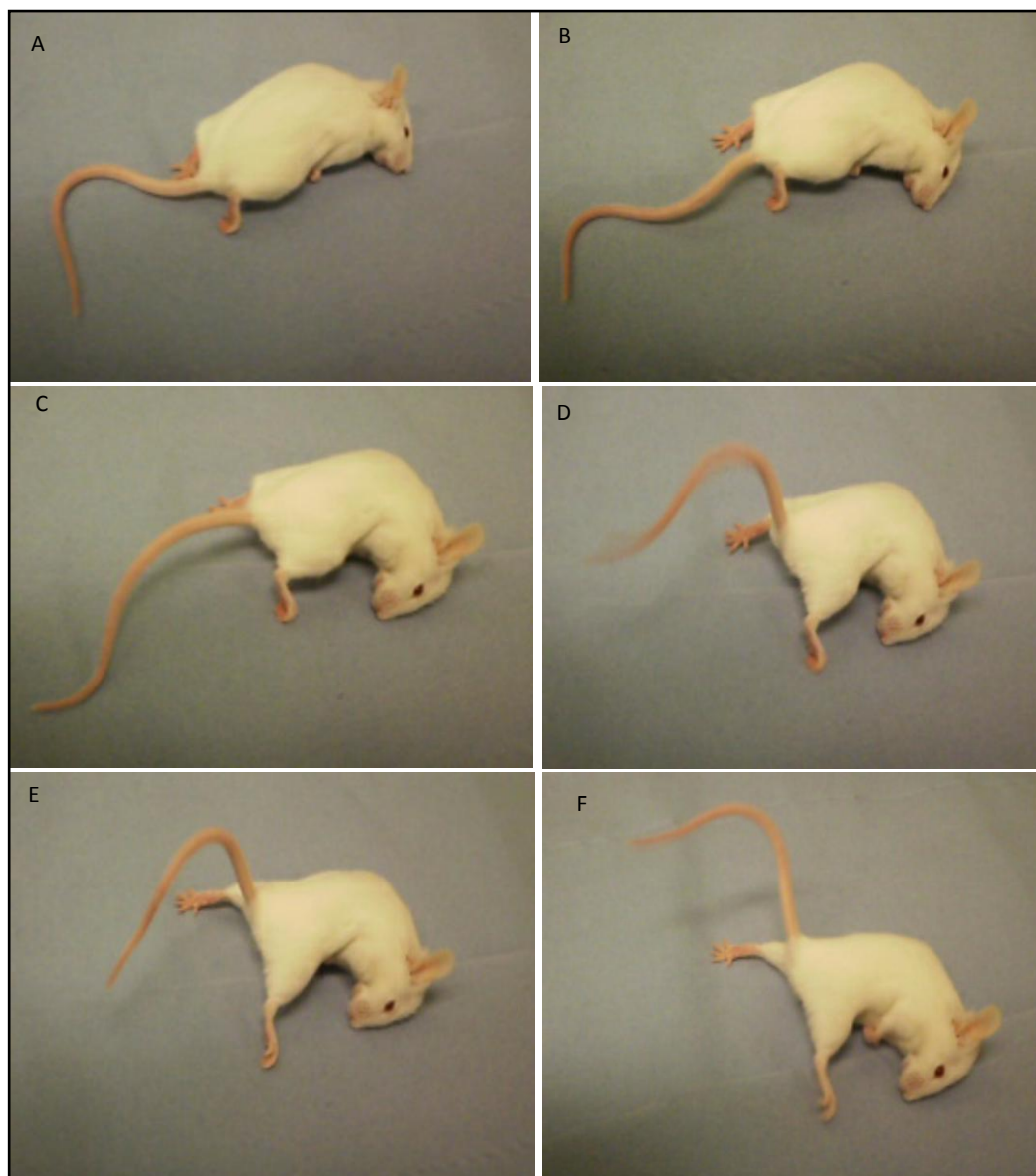


Figure 3.8: Picture frames of ABH mouse injected with EL IgG (0.5 mg/mL). Images A to F show individual picture frames from a video of an ABH mouse injected intra-cerebrally with 30 μ L of purified EL IgG (0.5 mg/mL). **(A)** 2 s. **(B)** 10 s. **(C)** 30 s. **(D)** 40 s. **(E)** 1 min. **(F)** 1 min 15 s.

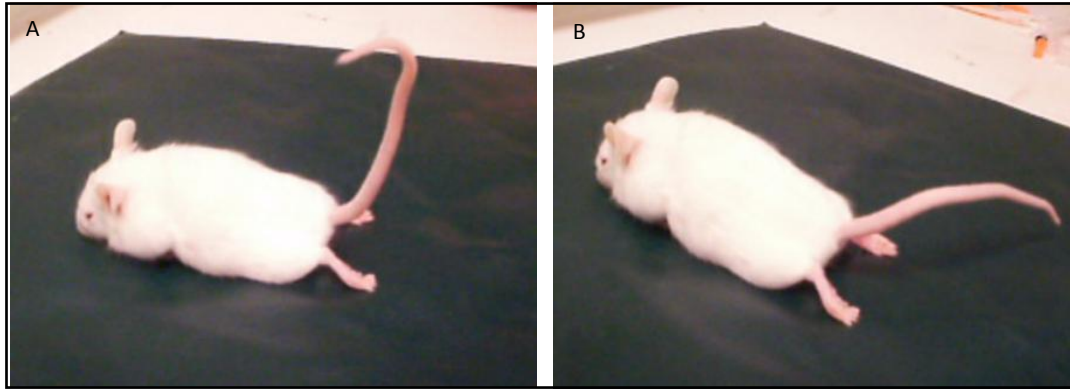


Figure 3.9: Picture frames of ABH mouse injected with EL IgG (0.05 mg/mL). Images A and B show individual picture frames from a video of an ABH mouse injected intra-cerebrally with 30 μ L of purified EL IgG (0.05 mg/mL). **(A)** 2 s. **(B)** 30 s.

3.5.2 Dystonia

Female Biozzi ABH mice were injected intra-cerebrally with a volume of 30 μ L of purified IgG from a patient diagnosed with dystonia at concentration of 0.1 and 1mg/mL. Three mice were used per experimental group. Hind limb rigidity and an abnormal, twisted posture due to sustained muscle contraction were observed in mice injected with 1 mg/mL of IgG. At 0.1 mg/mL the animal remained motionless during the course of the experiment. The symptoms developed in the mice injected with autoantibodies from a dystonia patient varied from those seen in the EL model. Hind limb rigidity was much more pronounced in these animals as opposed to tail rigidity seen in the EL group.

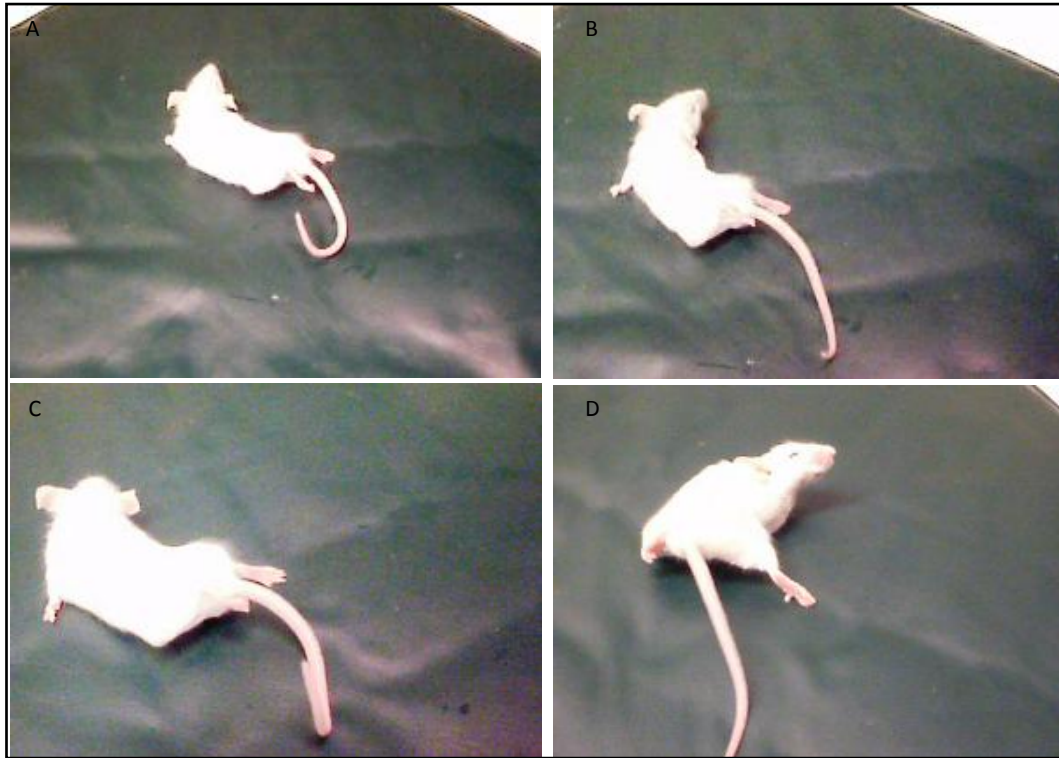


Figure 3.10: Picture frames of ABH mouse injected with dystonia IgG (1 mg/mL). Images A-D show individual frames from a video of an ABH mouse injected intra-cerebrally with 30 μ L of purified IgG from a patient diagnosed with dystonia (1 mg/mL). **(A)** 2 s. **(B)** 10 s. **(C)** 30 s. **(D)** 1 min.

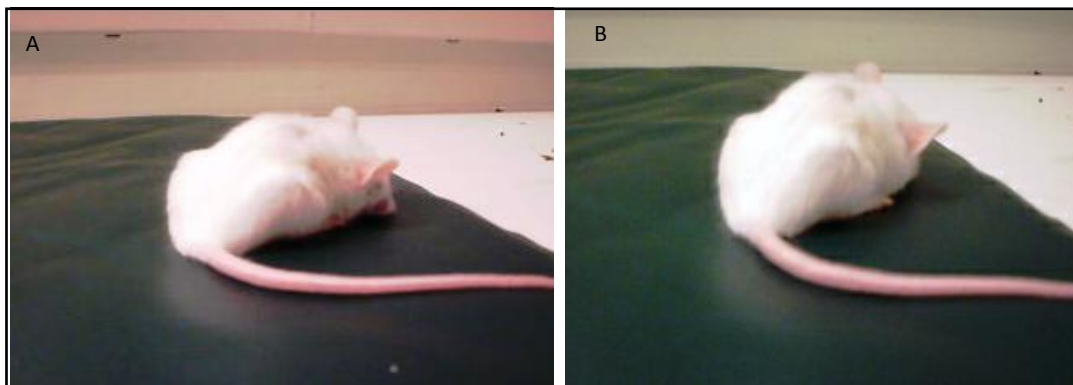


Figure 3.11: Picture frames of ABH mouse injected with dystonia IgG (0.1 mg/mL). Images A and B show individual picture frames from a video of an ABH mouse injected intra-cerebrally with 30 μ L of purified IgG from a patient diagnosed with dystonia (0.1 mg/mL). **(A)** 2 s. **(B)** 30 s.

3.5.3 Mouse mAb against NSE

Female Biozzi ABH mice were injected intra-cerebrally with a volume of 30 μ L of mAb against enolase at concentration of 0.1 and 1mg/mL. This was carried out to confirm that enolase is indeed a target autoantigen in this group of disorders. At a concentration of 0.1mg/kg the animals developed severe signs of rigidity and tremor and showed no signs of recovery. Although less severe both these classical symptoms of neuropsychiatric movement disorders were still observed in animals injected with the antibody diluted to 0.01mg/mL.

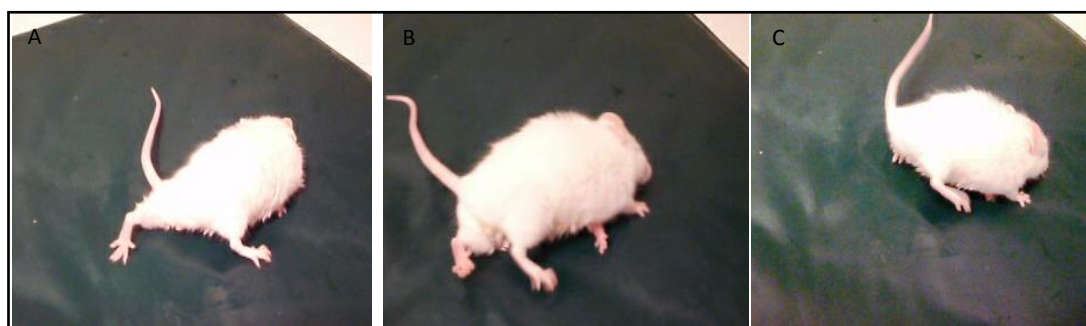


Figure 3.12: Picture frames of ABH mouse injected with mAb against NSE (0.5 mg/mL).

Images A to C show individual picture frames from a video of an ABH mouse injected intra-cerebrally with 30 μ L of mAb against NSE (0.5 mg/mL). (A) 2 s. (B) 20 s. (C) 1 min 15 s.

3.5.4 Healthy Controls

Female Biozzi ABH mice were injected intra-cerebrally with a volume of 30 μ L of purified IgG from a healthy control at concentration of 0.1 and 1mg/mL. Three mice were used per experimental group. The mice showed no signs of rigidity, tremor or any other signs of distress as observed with the mice injected with patient IgG. Once the animal had recovered from the anaesthesia it showed normal behavioural and locomotor activity

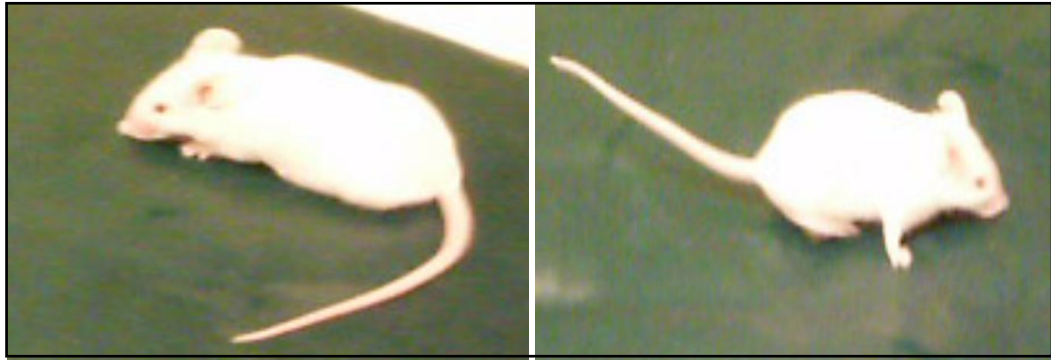


Figure 3.13: Picture frames of ABH mouse injected with healthy control IgG (1 mg/mL).

Images A and B show individual picture frames from a video of an ABH mouse injected intra-cerebrally with 30 μ L of IgG purified from healthy control sera.. (A) 20 s. (B) 1 min.

3.6 DISCUSSION

Animal models are a potential tool for developing or testing novel pharmacological therapies. They facilitate the study of genetic and environmental factors that contribute to the expression of behavioural and locomotor deficits which are the two most important parameters that help to define the phenotypes of mice.

In the active immunization model the open field test conducted helped systematically assess a range of parameters like novel environment exploration, general locomotor activity, and an initial screen for anxiety-related behaviour in rodents (Prut et al., 2003). However, behaviour during the test can be influenced by genetic variation, sex, age and exposure to illumination in the chambers. Excess noise and human activity during testing period, excess odours in the room, and distracting visual signals were controlled to minimize variation in data collection. Animals with decreased muscle function will be less active (decreased horizontal and vertical activity, decreased movement time, and increased rest time), and the total distance travelled will be decreased, giving a sense of the level of ambulatory activity (Zhuang et al., 1999). Both SJL and ABH strains of mice showed a significant decrease in total distance travelled when injected with GABHS proteins as compared to CFA only controls. This could point towards neurobehavioral and locomotor abnormalities. It can also be indicative of anxiety-related behavior. Rearing and centre-perimeter residence time are used as measures of anxiety and it is assumed that the mice feel safer in the perimeter regions of the open field chambers, close to the walls (Treit and Fundytus, 1988; Carli et al., 1989; Meng and Drugan, 1993; Steiner et al., 1997; Angrini et al., 1998; Heisler et al., 1998; Nasello et al., 1998). More ventures or exploratory excursions into the centre of the chamber are therefore interpreted as a decrease in anxiety (Schamm et al., 2001). It has been suggested that two factors influence anxiety-like behaviour in the open field. The first is social isolation resulting from the physical separation from cage

mates when performing the test. The second is the stress created by the brightly lit, unprotected, novel test environment (File, 1980; Prut et al., 2003). The measure of anxiety has been validated predictively by the demonstration that known anxiolytic agents (specifically diazepam, chlordiazepoxide, and pentobarbital) dose dependently decrease the amount of time spent in the perimeter of the open field (Treit and Fundytus, 1988), and genetically modified mice lacking particular 5-HT receptor subtypes (Heisler et al., 1998; Ramboz et al., 1998; Zhuang et al., 1999) or D3 dopamine receptor (Steiner et al., 1997) show the expected change in centre perimeter residence time based on their demonstrated roles in mediating or decreasing anxiety. Definitive conclusions can only be made by conducting additional behavioral tests.

In the present study the mice were subjected to rotarod assessment and the length of time that the animal stays on the rotating rod is a measure of their balance, coordination, physical condition, and motor-planning. I failed to find any difference in motor coordination between mice immunized with GABHS or recombinant proteins and CFA controls. This contradicts the findings by Yaddanapudi et al. (2009) and Brimberg et al. (2012) where they depicted that GABHS immunized mice/rats remained on the rotorod for a shorter interval of time compared to PBS controls (Yaddanapudi et al., 2009; Brimberg et al., 2012).

Out of the four speculated autoantigen enolase has been found to be the most prominent putative autoantigen in the literature (Dale et al., 2002). In our study higher sera reactivity was observed against γ -enolase as compared to α -enolase and ald C. This coincides with patient studies where most OCD sera tested positive for ABGA had antibodies specifically against enolase (Nicholson et al., 2012). Previous studies have reported the presence of enolase-specific autoantibodies in a range of psychiatric, degenerative and inflammatory

disorders, including discoid lupus erythematosus, autoimmune polyglandular syndrome type 1 primary and secondary membranous nephropathy, cancer-associated retinopathy, autoimmune hepatitis type 1, mixed cryoglobulinemia with renal involvement, cystoid macular oedema, endometriosis (Gitlits et al., 2001; Witkowska et al., 2005). Based on the results from the animal model and previous findings, the development of an enolase enzymatic assay was attempted in order to test patient sera for antibodies against enolase.

H&E staining showed infiltration in the brain tissue recovered from ABH mice. IgG deposits in several brain regions, including deep cerebellar nuclei (DCN), globus pallidus, and thalamus have been observed when sera from GABHS-immunized mice were tested for immunoreactivity to mouse brain (Hoffman et al., 2004). Similar findings have been reported by Brimberg et al. (2012) where once again streptococcal exposure in rats resulted in antibody deposition in the striatum, thalamus, and frontal cortex consistent with the known pathophysiology of SC and related neuropsychiatric disorders (Brimberg et al., 2012). Delineation of the characteristics of these autoantibodies like analysing the Ig class/subclass, specificity and affinity for specific epitopes in the CNS is critical in defining the pathogenesis immune-mediated neuropsychiatric syndromes.

The science of neuropsychiatric movement disorders specially TS is advancing at multiple levels of analysis and is being enhanced through the use of animal models. Particularly hard challenges in the development of animal models of these disorders reflect its complex features. The lack of detailed behavioural testing in our study prevented us from picking-up behavioural and locomotor abnormalities reminiscent of these disorders. It is planned to develop this animal model further by injecting mice with purified M proteins (including specific serotypes) or/and superantigens which would help us establish a link between streptococcus and these disorders together with pin pointing the proteins causing the

virulence. Also the immunological effects of superantigens are generally species specific, therefore GABHS superantigens may need to be replaced with superantigens known to affect mice. Mice challenged with superantigens primed with bacterial lipoproteins have showed higher levels of serum TNF- α and IL-6 compared to those exposed to either agent alone (Kearney et al., 2011). Also a number of behavioural tests need to be set up to better evaluate these animals. In addition subtle changes in stereotypies need to be picked up for which I plan to use blinded video tape analysis of these animal. These animal models may translate directly to human disease and may prove helpful in discovering autoantibody targets in TS and other postulated streptococcal-related neuropsychiatric disorders.

Animal models might not be able to predict the immunogenicity of antigenic/therapeutic proteins in humans reliably. The mechanisms underlying immunogenicity differ between conventional animal models and humans, hence the value of these models is limited and studies have shown that conventional animal models over-estimate immunogenicity in patients (Koren et al., 2002). In non-human primate a variety of responses ranging from weak, no antibody response, to a strong neutralizing response, or even a cross-reactive antibody response have been observed (Bussiere, 2003). These might not always correlate well with the immune response that is seen in humans. However, for proteins that are conserved across species, non-human primates might have some predictive value like enolase, an enzyme that presents a high evolutionary conservation among species (Carneiro et al., 2004; Pal-Bhowmick et al., 2007).

Additionally the duration and schedule of dosing, route of administration, dosage of the protein, pharmacological properties of the protein, as well as the purity of the clinical material can influence its immunogenicity (Brinks et al., 2011). The use of transgenic mice or homologous proteins can be used to predict neo-epitopes, relative immunogenicity, and

breaking of tolerance. However, they are less suitable to predict the incidence of immunogenicity or clinical effect of antibody formation. HLA transgenics might serve as an interesting tool to study whether the immunogenic response is HLA restricted (Van Beers et al., 2010; Brinks et al., 2011).

An important question is whether or not anti-neuronal antibodies are directly involved in the pathogenesis of the neuropsychiatric disorders or simply diagnostic markers. Two studies investigating the effects of infusing serum immunoglobulin from patients with PANDAS into rat striatum found an increase in stereotypical movements compared to control antibodies (Hallett et al., 2000; Taylor et al., 2002). However, another group using the same methods failed to reproduce the results (Loiselle et al., 2004) and more recently a collaborative study was also reported as being negative (Singer et al. 2005) although this study has been criticised for being methodologically flawed (Giovannoni, 2005).

The present study is the first of its kind to create an intra-cerebral animal model of neuropsychiatric disorders like EL. The development of signs in mice after direct injection of antibodies representative of the disease in question confirms the pathogenicity of these antibodies. According to *Witebsky's postulates* (formulated by Ernst Witebsky and colleagues in 1957) (Rose and Bona, 1993) a disease to be regarded as an autoimmune disease has to fulfil the following three essential criteria (i) Direct evidence from transfer of pathogenic antibody or pathogenic T cells (ii) Indirect evidence based on reproduction of the autoimmune disease in experimental animals (iii) Circumstantial evidence from clinical clues. The animal model fulfils the first two criteria listed above. The clinical evidence of autoimmunity is subject to interpretation and may be fulfilled by evidence from a controlled trial of treatment with either plasma exchange or IVIg in children with PANDAS that demonstrated a significant improvement in motor and psychiatric symptoms for both

therapies compared to placebo (Perlmutter et al., 1999). These observations and insights from the proposed treatment effects of IVIG suggest that the autoantibodies are pathogenic in these disorders. In summary EL and other ABGA-associated disorders are an emerging entity, with major implications for neuropsychiatry. Establishing this group of disorders as “true” autoimmune diseases marks the first step towards understanding their pathogenesis which would in turn have therapeutic implications.

The spectrum of post-streptococcal CNS disorders includes tics, chorea and dystonia (Church et al., 2004; Edwards et al., 2004). However the symptoms associated with each of these disorders is variable. EL symptoms include high fever, headache, double vision, delayed physical and mental response, tremors and lethargy. In acute cases, patients may enter a coma like state termed akinetic mutism (Dale et al., 2004; Lopez-Alberola, 2009). On the other hand symptoms of dystonia include muscle spasms, abnormal muscle tone leading to progressive rigidity (Madhusudanan, 1999). Although part of the same disease spectrum, the different effect of these antibodies may be due to variable cellular and/or sub-cellular targets in this group of disorders.

In the present study I wanted to determine the specificity of ABGA and hence compare the effects of antibodies from both EL and dystonia patients. A varied phenotype was observed in the all three cohorts with some common features, in particular rigidity. A less severe disease phenotype was reproducible in animals injected with a very low antibody concentration that enabled us to observe subtle phenotypical changes as opposed to a more pronounced effect observed with higher antibody concentration. In order to confirm whether enolase was one of the target autoantigen in this group of disorders antibody mAb against NSE was injected in mice. Injection of mAb against NSE did result in a phenotype very similar to that seen in mice injected with patient antibodies exhibiting classical signs of

tremor and rigidity. This effect again was reproducible even at low antibody concentrations. The preliminary results of this experiment point towards NSE being at least one of the antibody targets in this spectrum of neuropsychiatric movement disorders, with functional effect when injected intracerebrally bypassing the blood-brain-barrier.

Given its novelty the present animal model forms the basis for a much larger set of experiments. Future work will involve injecting animals specifically in the basal ganglia which can be achieved by the use of a stereotactic frame. Additionally, antibodies from patients with TS could be used to see whether stereotypies and episodic utterances can be detected that have been proposed as a model of motor and vocal tics. Also it would be interesting to determine if the use of affinity purified antibodies against the known autoantigens especially enolase from sera of patients will induce effects comparable with the commercial monoclonal antibody. Furthermore, electrophysiological experiments would be carried out to rule out the occurrence of epileptic seizures and confirm that the stereotypical movements seen in the mice are indeed representative of the disorders in question.

All the above findings point towards a possible autoimmune origin of neuropsychiatric movement disorders that will have implications for both our understanding of the pathophysiology of these disorders and future therapeutic strategies. Future experiments should refine this existing animal model to create an animal model that replicates the behavioural, pharmacological, and immunological abnormalities seen in this group of neuropsychiatric disorders.

CHAPTER 4

FUNCTIONAL ASSAYS

4.1 INTRODUCTION

4.1.1 Effect of Autoantibodies on Neuronal Function

An important question is whether or not anti-neuronal antibodies detected in postulated post-streptococcal neuropsychiatric disorders are directly involved in the pathogenesis of these disorders or are simply a diagnostic marker. As described earlier these autoantibodies have been shown to recognize four main protein bands of 40, 45, 60 and 98kDa which have been identified aldolase C (neurone specific), α and γ -enolase and pyruvate kinase (Dale et al., 2006). All three of the major candidate autoantigens proposed in this group of postulated post-streptococcal disorders have protein homologues in streptococci. Various hypotheses on the mode of action of these antibodies have been made based on the discovery of receptor or channel coupling of the antigens. Tests on cerebellar granular cells (CGC), have shown that incubation with commercial antibodies against the identified antigens enolase, pyruvate kinase and aldolase C increase the rate of apoptosis (Dale et al., 2006).

Interestingly, streptococcal enolase is also found on the surface of the bacterium and appears to function as an efficient plasmin(ogen) binding protein which influences tissue invasiveness and pathogenicity (Pancholi and Fischetti, 1998). The streptococcal surface enolase antibodies appear to recognise a shared epitope with neuronal surface and cytoplasmic enolase. It was hypothesized that these antibodies may have a direct effect on neuronal function. Membrane neuronal aldolase provides local membrane energy and is enzymatically active (Bulliard et al., 1997). It also forms an oxidoreductase complex with enolase and other proteins on the neuronal membrane and is thought to monitor oxidative

stress and induce an appropriate cellular response (Bulliard et al., 1997). Aldolase binds tightly with ATPase protein pumps on the plasma membrane allowing direct coupling of glycolysis to the proton pump (Lu et al., 2001). The monomer of pyruvate kinase acts as thyroid hormone (T3) binding protein. Binding of T3 to pyruvate kinase inhibits enzymatic activity, suggesting that this process may be centrally involved in the control of some cellular metabolic effects induced by thyroid hormones (Kato et al., 1989). Interestingly, hyperthyroidism is a well-described cause of chorea. Membrane glycolysis provides a preferential source of ATP in order to maintain myocyte K^+ channels (Weiss & Lamp, 1987), ATPase and calcium uptake (Hardin et al., 1992) and Na^+/K^+ pumps on intestinal cells (Dubinsky et al., 1998). The maintenance of these pumps may be directly linked to functionally compartmentalised ATP to ADP ratios on the cell membrane (Dubinsky et al., 1998).

Since all the above antigens are glycolytic enzymes involved in the preferential source of ATP, apoptosis was suggested to occur as a result of energy failure at the cell surface (Dale et al., 2006). Stress leads to an initiation of intracellular apoptotic signalling process in cells. The binding of nuclear receptors by heat, radiation, glucocorticoids, nutrient deprivation, infection, hypoxia and increased intracellular calcium concentration for example, can trigger the release of intracellular apoptotic signals by a damaged cell (Mark et al., 2003). Ca^{2+} influx into cells activates enzymes, including phospholipases, proteases and endonucleases leading to damage of structures such as DNA and the cytoskeleton (McConkey and Nutt, 2001; Gronski et al., 2009). Cytoplasmic levels of Ca^{2+} can increase either from release from internal calcium stores such as the endoplasmic reticulum (ER) or entry from outside the cell via calcium channels, leading to the rapid activation of molecules that promote activation, proliferation and other functions in a cell (McConkey and Nutt, 2001). Mitochondria also are damaged by excessive surges of calcium (Gronski et

al., 2009). Mitochondria utilise the extra calcium and eventually swell and cease functioning (Gronski et al., 2009). Sodium channels are also disrupted at the time, aggravating and increasing the eventual cell death. Phagocytes are also known to utilize calcium flux. Activation of macrophages using LPS is shown to cause an increase in cytosolic calcium and the eventual production of TNF- α (Watanabe et al., 1996; Cuttall et al., 2008).

In summary membrane glycolytic enzymes are closely involved in the energy provision and maintenance of ion channels on the neuronal membrane, trophic support and other functions. Therefore disrupting their activity is likely to lead neuronal dysfunction.

4.2 HYPOTHESIS AND AIMS

Serologic studies of children with post-streptococcal neuropsychiatric disorders have detected anti-neuronal antibodies but their role in the disease has not yet been explored. I hypothesized that these autoantibodies will have functional effects *in vitro*.

The main aim of this study was to investigate the functional effects (cytotoxicity, apoptosis, intracellular calcium flux) of anti-neuronal antibodies from patients diagnosed with postulated post-streptococcal disorders like TS, EL, SC and dystonia on both non-neuronal and neuronal cells.

4.3 MATERIALS AND METHODS

4.3.1 Clinical Samples

Serum samples from a mixed cohort of patients diagnosed with TS, EL, SC and dystonia were used in cytotoxicity, apoptosis and calcium flux assays as described below. Patient material was obtained with informed consent and appropriate ethical review.

The samples used in this study were collected from Tertiary referral centres, Great Ormond Street Hospital (GOSH) and National Hospital of Neurology and Neurosurgery (NHNN),

therefore clinically they were in the severe end of spectrum. Consent for autoantibody and strep studies was obtained from the patients by Dr. R Dale (GOSH, now Sydney Children's Hospital, Australia) Prof. G Giovannoni (ION, now Royal London Hospital), Prof. N Quinn (NHNN) and Prof. M Robertson (NHNN). These samples were all known ABGA positive by Western Blot according to Church et al., 2002. They were all ASOT and/or DNaseB positive by Behring Nephelometer (Siemens). Throat culture was not routinely performed as they were referral patients.

The samples used were anonymized and stored at -20°C. They were typical positives in each clinical group that had sufficient volume of left to perform further experiment. There was nothing atypical about them regarding age, sex and streptococcal status.

Chorea: Patients were referred between 1999 and 2002 by their general practitioner or paediatrician for investigation and management of an acute onset or relapsing movement disorder. All patients had disease onset shortly after streptococcal pharyngeal infections. Movement disorders were initially diagnosed by Dr. Russell Dale. Movement disorders were video-recorded and reviewed by expert child neurologists (Dr. Robert Surtees and Dr. Brian Neville, GOSH) to validate the movement disorder classification. Patients were diagnosed clinically using clinical scores i.e using Jones's criteria for rheumatic fever (Jones et al., 1935; Jones, 1944) and chorea (evidence of streptococcal infection).

Tourette's: All TS patients were diagnosed by Prof. M. Robertson using standardised instruments, including the National Hospital Interview Schedule (Robertson et al., 1996), the Diagnostic Interview Schedule (Robertson et al., 1999) and the Yale Global Tic Severity Rating Scale (Leckman et al., 1989). In order to make a diagnosis of TS, patients had to satisfy DSM-IV-TR (APE, 2000) and ICD-10 (WHO, 1992) criteria. Thus all patients had to have multiple motor and one or more vocal tics, with symptoms lasting longer than 1 year.

Consent was obtained for each patient by Prof. Robertson and 1, 10mL, clotted blood sample was taken, centrifuged on the same day at 2000rpm for 10 minutes and stored in 2 aliquots at -20°C.

Encephalitis Lethargica: All patients were referred to tertiary neurology centers between April 1999 and May 2002 with a new-onset CNS dysfunction resulting in an EL-like syndrome (sleep disorder and associated lethargy, parkinsonism and neuropsychiatric disorders). Movement disorders were video-recorded and reviewed by expert child neurologists (Dr. Robert Surtees and Dr. Brian Neville, GOSH). Psychiatric disturbances were often acute analyzed through DSM-IV criteria .

Dystonia: Dystonia patients all came from the Movement disorder clinic at the National Hospital for Neurology and Neurosurgery, diagnosed by Prof. N Quinn and Prof. K Bhatia (ION). The cases were identified during an ongoing study evaluating the prevalence of ABGA (Church et al., 2002). Generalized dystonia was observed in all patients.

Healthy Controls: Serum from healthy controls were provided by Dr. Ruth Dobson at the Royal London Hospital, Queen Mary University of London.

Cohorts	Centre	Age Range (Mean)	Sex Ratio (F:M)	Publication
Chorea	Great Ormond Street Hospital for Children(GOSH), London. Institute of Neurology (ION), UCL.	1.3 – 13 yrs (7.15)	2:1	Dale et al., 2004
Tourette's Syndrome	University of Bari, Italy. National Hospital for Neurology and Neurosurgery (NHNN), London.	6- 14 yrs (10.4)	1:5	Dale et al., 2004
Encephalitis Lethargica	Great Ormond Street Hospital for Children(GOSH), London. Institute of Neurology (ION), UCL. The Royal London Hospital	2- 69 yrs (17.85)	1.2:1	Dale et al., 2004
Dystonia	Institute of Neurology (ION), UCL.	17- 57 yrs (39.75)	4:1	Edwards et al., 2004
Healthy Controls	The Royal London Hospital	22- 50	4:1	N/A

Table 4.1: Clinical serum samples used for the functional assays in this study. The table above summarises the clinical cohorts used in the present study in terms of collection centre, age range and female: male (F:M) sex ratio. Clinical details of all samples have been previously published.

In addition sera from ABH and SJL mice immunized with GABHS proteins and recombinant proteins (Ald C, enolase and pyruvate kinase) were also used for all the assays to compare their functional effects on cell function as opposed to CFA only immunized control animals.

All assay were carried out using purified IgG from sear from patients , controls And animal models as described on page 118.

4.3.2 PC12 cells

Background:

PC12 is a cell line derived from a pheochromocytomais (neuroendocrine tumor) of the rat adrenal medulla (Greene and Tischler, 1976). When exposed to neurotrophins like the nerve growth factor (NGF) neoplastic PC12 cells undergo a series of biochemical and morphological changes thereby differentiating into neural cells (Walters et al., 2002).

PC12 cells grown with or without NGF contain dense core chomaffin-like granules up to 350 nm in diameter. The NGF-treated cells also contain small vesicles which accumulate in process varicosities and endings. PC12 cells synthesize and store the catecholamine neurotransmitters dopamine and norepinephine (Cunningham et al., 2001).

PC12 cells were cultured in T75 culture flasks coated with Rat Tail Collagen I (Invitrogen Life Technologies, UK). The cells were maintained in RPMI 1640 medium (Sigma-Aldrich, Poole, Dorset, UK) supplemented with 50mL Horse serum (Lonza 14-403F), 25mL foetal calf serum (Sigma-Aldrich, Poole, Dorset, UK), 5mL penicillin/streptomycin (Sigma-Aldrich, Poole, Dorset, UK) and 5mL non-essential amino acid solution (Sigma-Aldrich, Poole, Dorset, UK) in a humidified incubator at 37°C and 4.5% CO₂. The cells were passaged every 2-3 days in a ratio of 1:5. To induce differentiation into neurons the cells were treated with 50 ng/mL of nerve growth factor (Invitrogen Life Technologies, UK) every 2 days for upto a week.

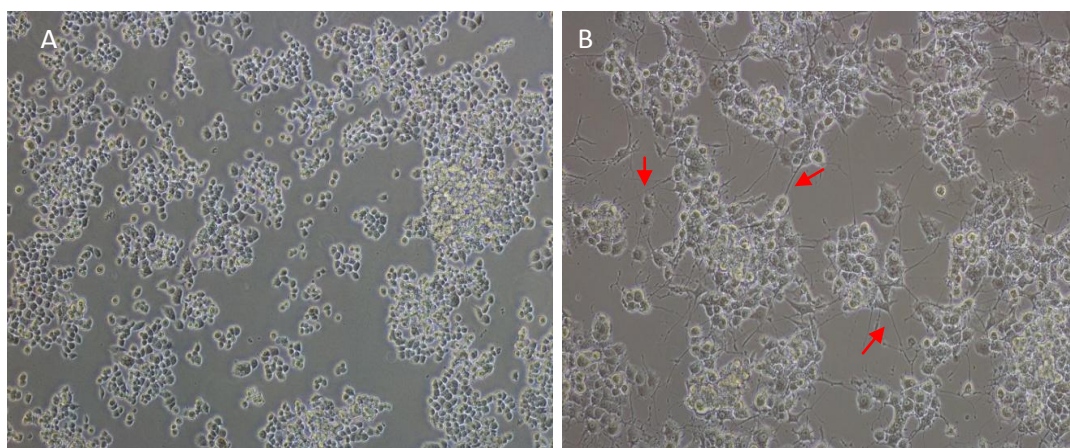


Figure 4.1: 40X light microscope images of PC12 cells before and after differentiation. (A) Undifferentiated PC12 cells. **(B)** PC12 cells after differentiation into neurons. These cells now show neurite growth (marked with an arrow) after 7 days of treatment with nerve growth factor (NGF).

4.3.3 Cytotoxicity Assay

WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) colorimetric cytotoxicity assay (Roche Applied Science, Basel, Switzerland) was performed on differentiated and undifferentiated PC12 cells probed with serum samples from TS (n=10), EL, SC, dystonia (n=18), healthy controls (n=19) and the animal model (CFA only (n=8), recombinant proteins (n=8), GABHS proteins (n=8). The stable tetrazolium salt WST-1 is cleaved to a soluble formazan by a complex cellular mechanism that occurs primarily at the cell surface. This bio-reduction is largely dependent on the glycolytic production of NAD(P)H present in viable cells. Therefore, the amount of formazan dye formed directly correlates to the number of metabolically active cells in the culture.

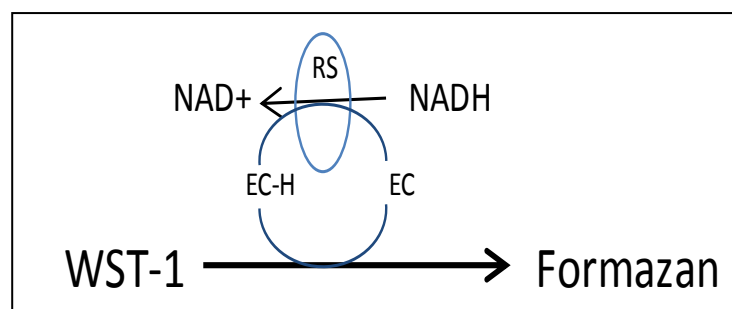


Figure 4.2: The biochemical reaction occurring in the WST-1 assay. Conversion of tetrazolium salt WST-1 into soluble compound formazan occurs through NAD(P)H which is only present in viable cells. Hence the amount of formazan formed directly correlates to the number of metabolically active cells in the culture.

1×10^4 cells (100 μ L/well) were grown in a 96-well tissue culture plate coated with rat tail collagen type I and incubated at 37°C for 24-48 h until desired confluency was achieved. Serum samples (1:100) were added to the cells and the plates were incubated for a period of 24 hours. 10 μ L of WST-1 reagent was then added to each sample and incubated at 37 °C under 7% CO₂ in a humidified incubator for variable time periods (0.5 h to 4 h). The WST-1 reagent incubation time of 1 h was found to be optimum since the color density did not change after this period. The formazan dye formed was quantitated with a scanning multi-well spectrophotometer (ELISA plate reader). The absorbance was measured at 450 nm with a reference wavelength of 620 nm. The delta optical density directly correlates to the number of viable cells. All experiments were done in duplicates.

4.3.4 Annexin V Apoptosis Assay

All FACS experiments were performed with Dr. Gary Warnes at the Flow Cytometry Core Facility, BICMS, Queen Mary, University of London.

Apoptosis in response to exposure with serum samples was performed using FITC Annexin V apoptosis detection kit 1 (BD Biosciences, Oxford, UK). Apoptosis is characterized by certain morphologic features, including loss of plasma membrane integrity, condensation of the cytoplasm and nucleus, and DNA fragmentation. In apoptotic cells, the membrane phospholipid phosphatidylserine (PS) is translocated from the inner to the outer leaflet of the plasma membrane, thereby exposing PS to the external cellular environment. Annexin V which is a 35-36 kDa Ca^{2+} -dependent phospholipid-binding protein binds to cells with exposed PS. Annexin V conjugated to fluorochromes like fluorescein isothiocyanate (FITC) thus serves as a sensitive probe for flow cytometric analysis of apoptotic cells. Since externalization of PS occurs in the earlier stages of apoptosis, FITC Annexin V staining identifies apoptosis at an earlier stage than assays based on nuclear changes such as DNA fragmentation. Mitochondria plays a pivotal role in apoptosis therefore, MitoTracker dye was used in conjunction with annexin V to track mitochondrial function in the cells. MitoTracker probes are cell-permeant mitochondrion-selective dyes that contain a mildly thiol-reactive chloromethyl moiety.

3×10^5 PC12 cells (undifferentiated and differentiated) were cultured onto collagen type I coated 24 well cell culture plates (1mL/well) and incubated for 48 hours at 37°C and 5% CO₂ in a humidified chamber. Serum samples from TS patients (n=10), other neuropsychiatric disorders like SC, EL and dystonia (n=10), healthy controls (n=10) and ABH and SJL animal model (CFA only (n=8), recombinant proteins (n=8), GABHS proteins (n=8)) diluted 1:100 were then added to each well and the cells were incubated for a further 24

hours. A pan-kinase c inhibitor, staurosporine (STS) (1µg/mL) was used as a positive control. Following the incubation the cells were scraped and transferred into FACS tubes. The cell pellets were then washed with 3mL of PBS without Ca²⁺ and centrifuged at 250 x g for 5 min. The supernatant was then discarded and the cell pellets were re-suspended in 100µl of PBS without Ca²⁺. A volume of 0.5µL of 1mM mitotracker dye (Invitrogen, UK) final concentration 40nM was added and the cells were incubated for 15 min at 37°C in a humidified chamber. The cells were again washed with 3mL of PBS without Ca²⁺ before being resuspended in 400µl of 1X Ca²⁺ rich buffer. 2µL of Annexin V- FITC (buffered in 50 mM Tris (pH 8.0) with 80 mM NaCl, 0.2% BSA, 1 mM EDTA, and 0.09% sodium azide) and 200ng/mL of propidium iodide staining solution (DAPI) was then added to all samples and incubated for 15 min at RT in dark. 10-20,000 events were then analyzed using BD LSRII flow cytometer (BD Biosciences, UK).

4.3.5 Calcium Flux Assay

Intracellular calcium flux in PC12 differentiated into neuronal cells when exposed to patient antibodies was determined using FACS. Intracellular calcium flux into the cell is representative of cell activation. The assay employed the use of Indo-1 which is a ratiometric calcium dye with a dual emission pattern: when bound to free intracellular calcium it emits at 390 nm (violet signal) and when unbound it emits at 530 nm (green signal) and the ratio of violet over the green signals is calculated as the ratio which is displayed as a parameter over time. Upon cell activation the dye should change from green to violet and hence the Indo-1 ratio should increase over time.

1x10⁵ PC12 cells/well were differentiated onto collagen type I coated 24 well cell culture plate. After differentiation the cells were scraped and transferred to BD Falcon™ round-bottom tubes (BD Biosciences, UK) followed by washes with PBS (-Ca²⁺) and centrifugation

at 270 x g for 5 min. The cell pellet was re-suspended in 1mL PBS (-Ca²⁺) to which 2µl of Indo-1 dye was added to achieve a final concentration of 2µM. The samples were then incubated for 45 min at 37°C in a humidified chamber. The samples were again washed with PBS (-Ca²⁺) and centrifuged at 270 x g for 5 min. The cell pellet was then resuspended in 1mL PBS (+Ca²⁺) and a baseline function level reading was taken for the first 30 seconds using BD FACS LSRII (BD Biosciences, UK). Serum samples from patients (n=8) healthy controls (n=8) and animal model (CFA only (n=3), RP (n=3), GABHS proteins (n=3)) at a dilution of 1:100 were then added to the suspension and each sample was read for a total of 5 min to see the changes in calcium flux. Ionomycin (10 µg/mL) was used as a positive control. Ionomycin is produced by the bacterium *Streptomyces conglobatus* and is used to raise the intracellular level of calcium (Yoshida and Plant, 1992). The data was analysed using the FlowJo v8.7.1 software.

4.4 RESULTS

4.4.1 Cytotoxicity Assay

4.4.1.1 Human Samples

WST-1 cytotoxicity assay was performed to analyze the effect of patient antibodies on both PC12 undifferentiated and PC12 differentiated into neurons. Sera samples from patients diagnosed with TS, SC, EL and dystonia were used for this assay along with healthy controls. The assay involved quantitation of cleaved of tetrazolium salt WST-1 to a soluble formazan. This bioreduction is dependent on the production of NAD(P)H which is present only in viable cells. Therefore, the amount of formazan dye formed directly correlates to the number of metabolically active cells in the culture. The absorbance was measured at 450 nm with a reference wavelength of 620nm. The delta OD directly correlates to the number of viable cells. A highly significant increase in cell cytotoxicity and decrease in the number of viable cells was observed in undifferentiated PC12 cells after exposure to antibodies from patients with TS and other neuropsychiatric movement disorders (SC, EL and dystonia) as compared to healthy controls ($p < 0.0001$). The higher rate of cell cytotoxicity after exposure to patient antibodies as compared to controls was also seen in PC12 cells differentiated into neurons ($p < 0.0001$).

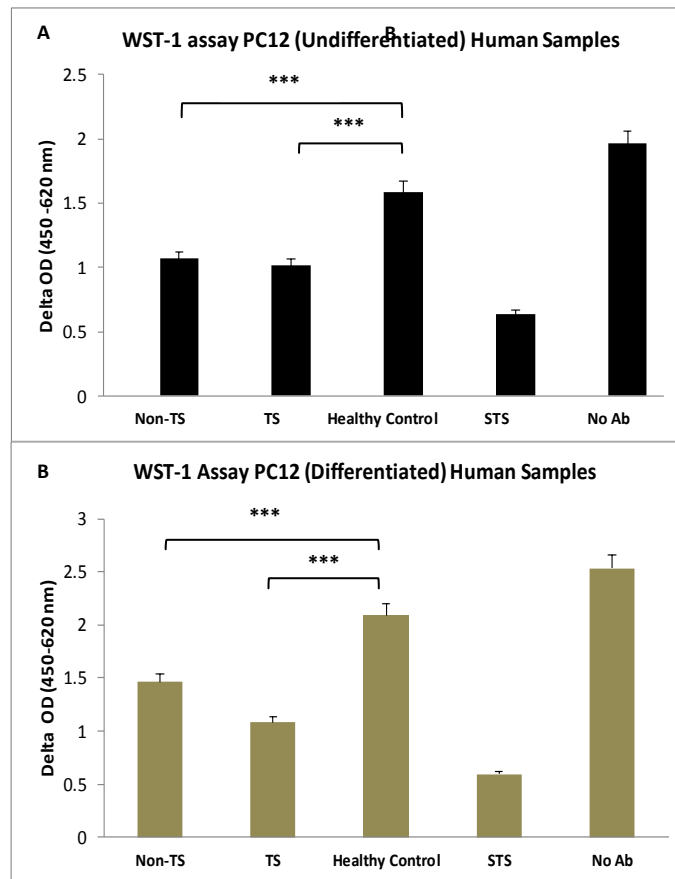


Figure 4.3: Measurement of cell viability (WST-1 cytotoxicity assay) in undifferentiated and differentiated PC12 cells using sera from patients and controls. Graph representing the cytotoxicity induced in undifferentiated and differentiated PC12 cells exposed to sera from patients diagnosed with TS and other neuropsychiatric disorders (SC, EL and dystonia) and healthy controls. The absorbance was measured at 450 nm with a reference wavelength of 620nm. The delta OD directly correlates to the number of viable cells. Staurosporine was used as a positive control. **(A)** A two tailed unpaired t-test revealed a significant decrease in the number of viable cells hence increased cytotoxicity rate in undifferentiated PC12 cells treated with sera from patients with TS ($p < 0.0003$) other neuropsychiatric disorders ($p < 0.0001$) as compared to healthy controls. **(B)** A two tailed unpaired t-test revealed a significant decrease in the number of viable cells hence increased

cytotoxicity rate in differentiated PC12 cells treated with sera from patients with TS ($p<0.0001$) other neuropsychiatric disorders ($p=0.0001$) as compared to healthy controls.

4.4.1.2 Animal Model Samples

WST-1 cytotoxicity assay was performed to analyze the effect of antibodies from SJL and ABH mice immunized with GABHS proteins and recombinant proteins (aldolase C, α -enolase, γ -enolase, pyruvate kinase) as compared to CFA only control mice on both PC12 undifferentiated and PC12 differentiated into neurons. As described earlier the absorbance was measured at 450 nm with a reference wavelength of 620 nm. The delta OD directly correlates to the number of viable cells.

A significant decrease in cell viability was observed in undifferentiated PC12 cells after exposure to antibodies from both ABH ($p<0.008$) and SJL ($p<0.004$) mice immunized with GABHS protein fractions. However, no difference in cell cytotoxicity was observed between sera sample from mice immunized with RP and CFA controls.

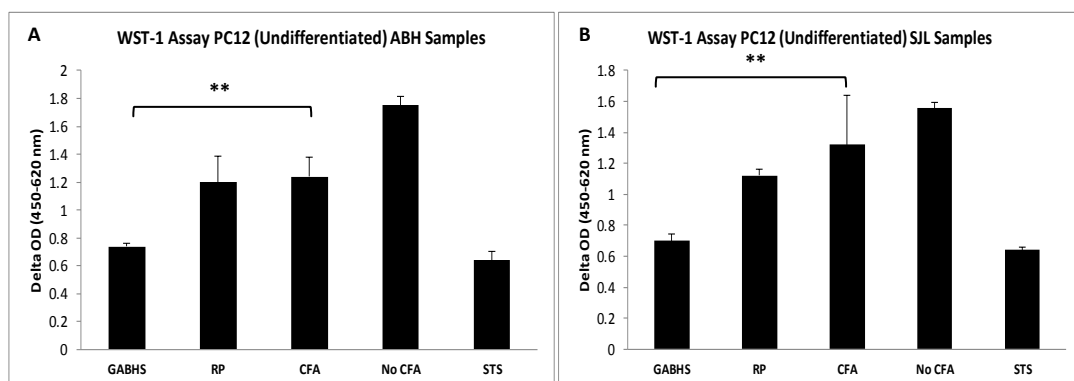


Figure 4.4: Measurement of cell viability in undifferentiated PC12 cells using sera from SJL and ABH animal models. Graphs representing the cytotoxicity induced in undifferentiated PC12 cells exposed to sera from ABH and SJL mice immunized with GABHS proteins, recombinant proteins (aldolase C, α -enolase, γ -enolase, pyruvate kinase). The

absorbance was measured at 450 nm with a reference wavelength of 620nm. The delta OD directly correlates to the number of viable cells. Staurosporine was used as a positive control. **(A)** A two tailed t-test revealed a significant decrease in the number of viable cells hence increased cytotoxicity rate in undifferentiated PC12 cells treated with sera ABH mice immunized with GABHS proteins ($p<0.008$) as compared to CFA only controls. **(B)** A two tailed t-test revealed a significant decrease in the number of viable cells hence increased cytotoxicity rate in differentiated PC12 cells treated with sera ABH mice immunized with GABHS proteins ($p<0.004$) as compared to CFA only controls.

A significant decrease in cell viability was observed in PC12 cells differentiated into neurons after exposure to antibodies from both ABH ($p<0.004$) and SJL ($p<0.005$) mice immunized with GABHS protein fractions. Additionally a highly significant decrease in cell viability was also seen in cells treated with antibodies from ABH mice immunized with RP ($p<0.009$). This effect was not observed with sera antibodies from SJL mice immunized with RP.

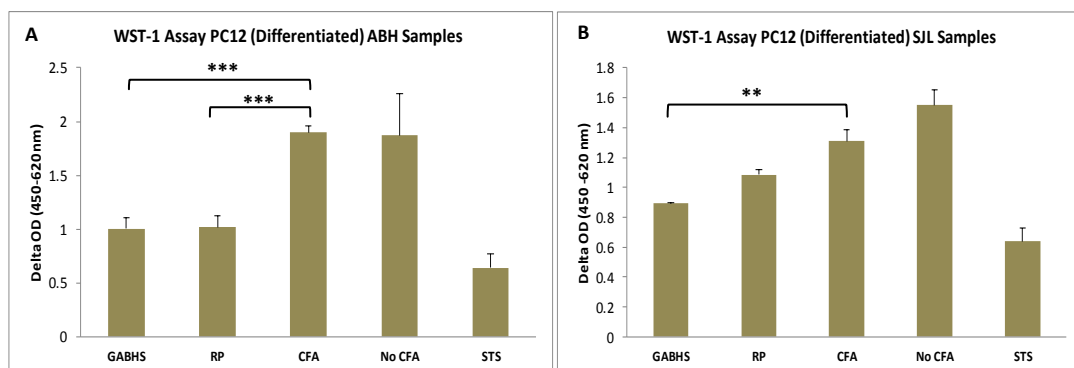


Figure 4.5: Measurement of cell viability in differentiated PC12 cells using sera from SJL and ABH animal models. Graphs representing the cytotoxicity induced in PC12 neuronal cells exposed to sera from ABH and SJL mice immunized with GABHS proteins, recombinant proteins (aldolase C, α -enolase, γ -enolase, pyruvate kinase). The absorbance was measured at 450 nm with a reference wavelength of 620nm. The delta OD directly correlates to the

number of viable cells. Staurosporine was used as a positive control. **(A)** A two tailed t-test revealed a significant decrease in the number of viable cells hence increased cytotoxicity rate in differentiated PC12 cells treated with sera ABH mice immunized with GABHS proteins ($p<0.004$) and recombinant proteins ($p<0.009$) as compared to CFA only controls. **(B)** A two-tailed t-test revealed a significant decrease in the number of viable cells hence increased cytotoxicity rate in differentiated PC12 cells treated with sera ABH mice immunized with GABHS proteins ($p<0.005$) as compared to CFA only controls.

4.4.2 Apoptosis Assay

4.4.2.1 Human Samples

An apoptosis assay involving measurement of Annexin V was performed on PC12 cells. This method is used to detect one of the earliest events in apoptosis—the externalization of phosphatidylserine (PS)—in living cells. Soon after apoptosis is induced, PS is translocated from the inner leaflet of the plasma membrane to the outer leaflet. FITC labelled Annexin V, has a strong and specific affinity for PS, to monitor the PS translocation that occurs due to apoptosis. Apoptosis was measured in both undifferentiated PC12 cells and cells differentiated into neurons after treatment with NGF after treatment with sera from patients with TS, EL, SC and dystonia and healthy controls. Staurosporin a known initiator of apoptosis was used a positive control. The results show a significant increase in apoptosis in undifferentiated PC12 cells after treatment with patient sera from TS patients ($p<0.01$) and those with other neuropsychiatric movement disorders ($p<0.0001$) as compared to healthy controls. Similar effect was observed with PC12 differentiated into neurons where antibodies from TS patients ($p<0.0001$) and those with other neuropsychiatric movement disorders ($p<0.0001$) caused a marked increase in apoptosis.

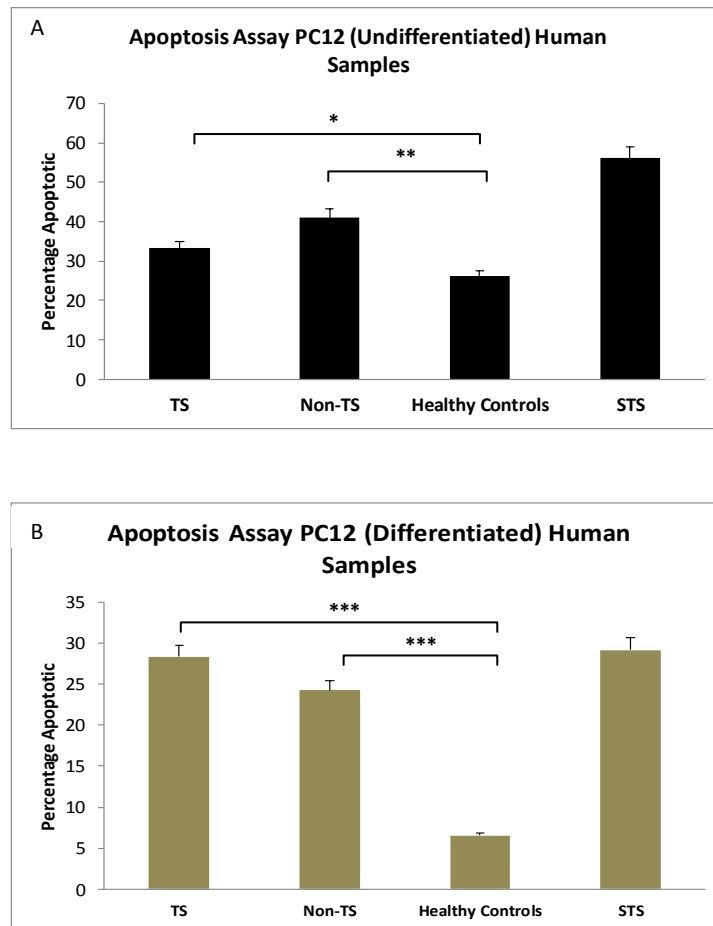


Figure 4.6: Measurement of cell apoptosis in undifferentiated and differentiated PC12 cells using sera from patients and controls. The graphs above depict the apoptosis induced in both undifferentiated and differentiated PC12 cells after the addition of sera samples from patients diagnosed with TS, other neuropsychiatric movement disorders (SC, EL and dystonia) as compared to healthy controls. The apoptosis assay involved measurement of Annexin V which detects one of the earliest events in apoptosis. Staurosporin was used as a positive control. **(A)** A two tailed t-test revealed a significant difference between the apoptosis induced by the addition of sera from patients with TS ($p < 0.01$) and non-TS disorders ($p < 0.0001$) as compared to healthy controls. **(B)** A two-tailed t-test showed a highly significant difference between apoptosis induced by the addition of sera from patients with TS ($p < 0.0001$) and non-TS disorders ($p < 0.0001$) as compared to healthy controls.

4.4.2.2 Animal Model Samples

An apoptosis assay involving measurement of Annexin V method to detect one of the earliest events in apoptosis—the externalization of phosphatidylserine (PS)—in living cells was performed. FITC labelled Annexin V, has a strong and specific affinity for PS, to monitor the PS translocation that occurs due to apoptosis. Apoptosis was measured in both undifferentiated PC12 cells and cells differentiated into neurons after treatment with sera from ABH and SJL mice immunized with GABHS proteins, RP (ald C, enolase and PK) and CFA as described in Chapter 3. The results from undifferentiated PC12 cells show a significant increase in apoptosis after treatment with sera from ABH immunized with GABHS and RP as opposed to mice immunized with CFA alone. However in case of SJL samples a significant increase in apoptosis is only observed after after treating the cells with sera from mice immunized with GABHS and not RP.

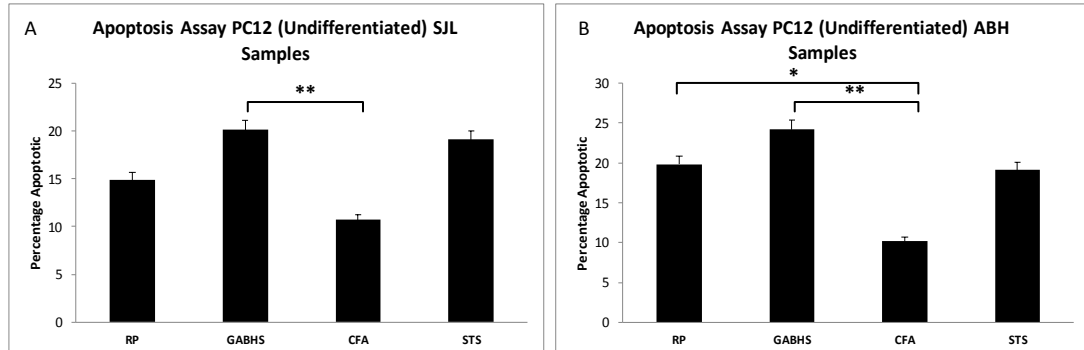


Figure 4.7: Measurement of cell apoptosis in undifferentiated PC12 cells using sera from SJL and ABH animal models. Graphs representing apoptosis induced in undifferentiated PC12 exposed to sera from SJL and ABH mice immunized with GABHS proteins, recombinant proteins (ald C, α - enolase, γ - enolase and pyruvate kinase) and CFA controls. The apoptosis assay involved measurement of Annexin V which detects one of the earliest events in apoptosis. Staurosporine was used as a positive control. **(A)** A two tailed t-test revealed a

significant increase in apoptosis in cells treated with sera SJL mice immunized with GABHS proteins ($p<0.008$) as compared to CFA only controls. **(B)** A two tailed t-test revealed a significant increase in apoptosis in cells treated with sera from ABH mice immunized with GABHS proteins ($p<0.0003$) and RP ($p<0.05$) as compared to CFA only controls.

A significant increase in apoptosis was observed in PC12 cells differentiated into neurons after the addition of sera from both SJL and ABH mice immunized with GABHS and RPs as compared to CFA only controls.

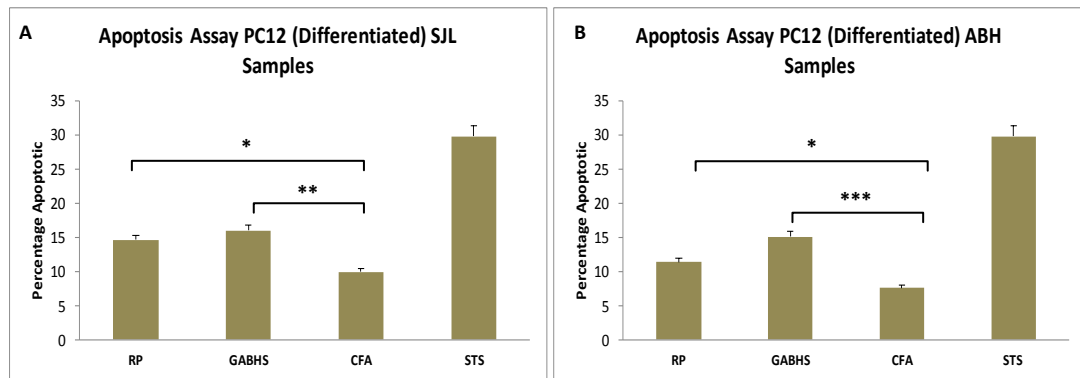


Figure 4.8: Measurement of cell apoptosis in differentiated PC12 cells using sera from SJL and ABH animal models. Graphs representing apoptosis induced in undifferentiated PC12 exposed to sera from SJL and ABH mice immunized with GABHS proteins, recombinant proteins (ald C, α -enolase, γ -enolase and pyruvate kinase) and CFA controls. The apoptosis assay involved measurement of Annexin V which detects one of the earliest events in apoptosis. Staurosporine was used as a positive control. **(A)** A two tailed t-test revealed a significant increase in apoptosis in cells treated with sera SJL mice immunized with GABHS proteins ($p<0.006$) and RPs ($p<0.02$) as compared to CFA only controls. **(B)** A two tailed t-test revealed a significant increase in apoptosis in cells treated with sera from ABH mice immunized with GABHS proteins ($p<0.0009$) and RP ($p<0.01$) as compared to CFA only controls.

4.4.3 Calcium Flux

4.4.3.1 Human Samples

Intracellular calcium flux that represents cell activation was measured in PC12 cells differentiated into neurons after being exposed to patient antibodies. The FACS assay was performed using Indo-1 which is a ratiometric calcium dye with a dual emission pattern i.e., when bound to free intracellular calcium it emits a violet signal and when unbound it emits green signal and the ratio of violet over the green is calculated as the Indo-1 ratio which is displayed as a parameter over time. Ionomycin was used as a positive control. The figures below show the real-time calcium flux into the cells after the addition of the sera samples from patients and healthy controls. PC12 neuronal cells exhibited a significant increase in intracellular flux after the addition of sera from patients with TS, SC, EL and dystonia as compared to healthy controls.

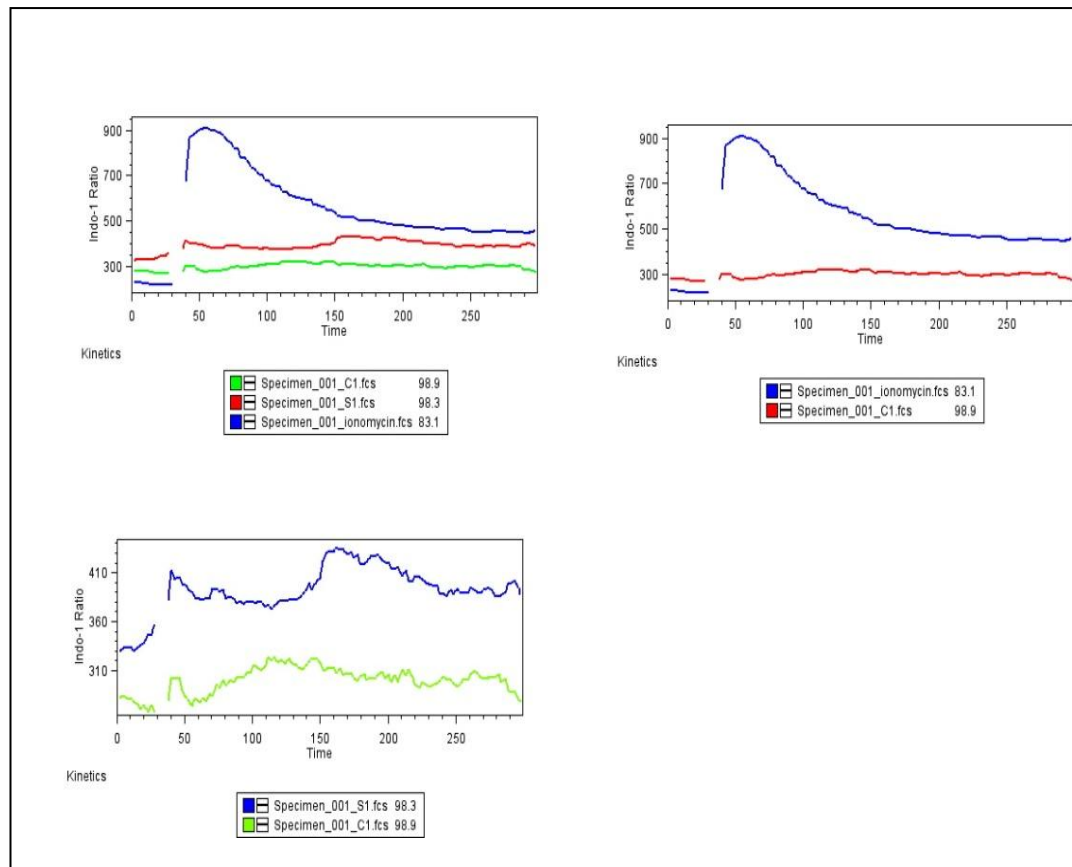


Figure 4.9: Real time intracellular calcium flux in differentiated PC12 cells after addition of of sera from patients and controls. The above image is illustrative of the real time changes in intracellular calcium levels in PC12 neuronal cells after the addition of sera from patients (S) and healthy controls (C). The FACS assay involved measurement of Indo-1 (calcium dye) ratio over time. Ionomycin was used as a positive control. A baseline measurement was done for the first 30 s after which the serum sample was added and the changes in intracellular calcium were measured for 5 min. An increase in calcium flux can be observed after the addition of ionomycin and sera samples at 30 s.

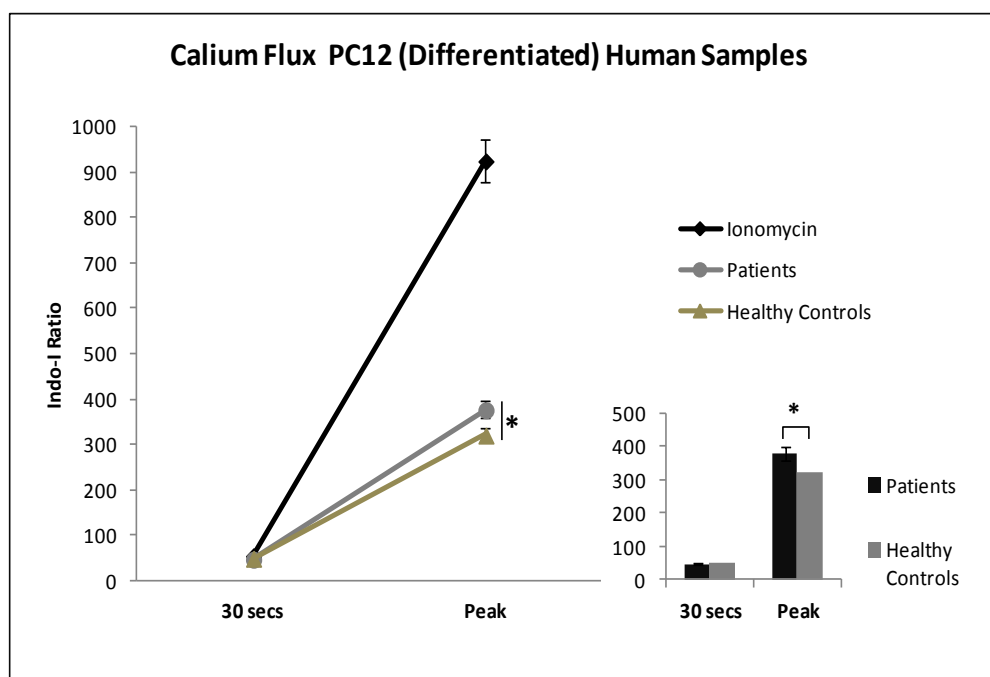


Figure 4.10: Intracellular calcium flux in PC12 neuronal cells following treatment with patient and control sera. The graph above depicts the real time intracellular calcium flux as a measure of Indo-1 ratio over time in PC12 neuronal cells after treatment with patient ($n=8$) and control ($n=8$) sera samples. A baseline reading was done for the first 30 s after which the serum sample was added and the changes in calcium flux monitored for 5 min. The Indo-1 ratio was measured just before (30 s) and after the addition of sera samples when it reached its peak. A two-tailed t-test revealed a significant increase in intracellular calcium flux after the addition of patient sera as opposed to healthy controls ($p<0.03$).

4.4.3.2 Animal Model Samples

Intracellular calcium flux that represents cell activation was measured in PC12 cells differentiated into neurons after addition of sera antibodies from active immunization animal model described in Chapter 3. The FACS assay was carried out using Indo-1 which is a ratiometric calcium dye with a dual emission pattern i.e., when bound to free intracellular calcium it emits a violet signal and when unbound it emits green signal and the ratio of violet over the green is calculated as the Indo-1 ratio which is displayed as a parameter over time. Ionomycin was used as a positive control. The figures below shows the real-time calcium flux into the cells after the addition of the sera samples from SJL and ABH immunized with GABHS proteins, RP (ald C, enolase and PK) and CFA only. PC12 neuronal cells exhibited an increase in intracellular flux after the addition of sera antibodies. The flux was significant when sera from SJL ($p < 0.02$) and ABH ($p < 0.04$) mice immunized with GABHS proteins was added to differentiated PC12 cells as compared to CFA only controls. The effect on calcium flux wasn't pronounced in response to sera from mice immunized with RPs (putative autoantigens) as compared to CFA controls.

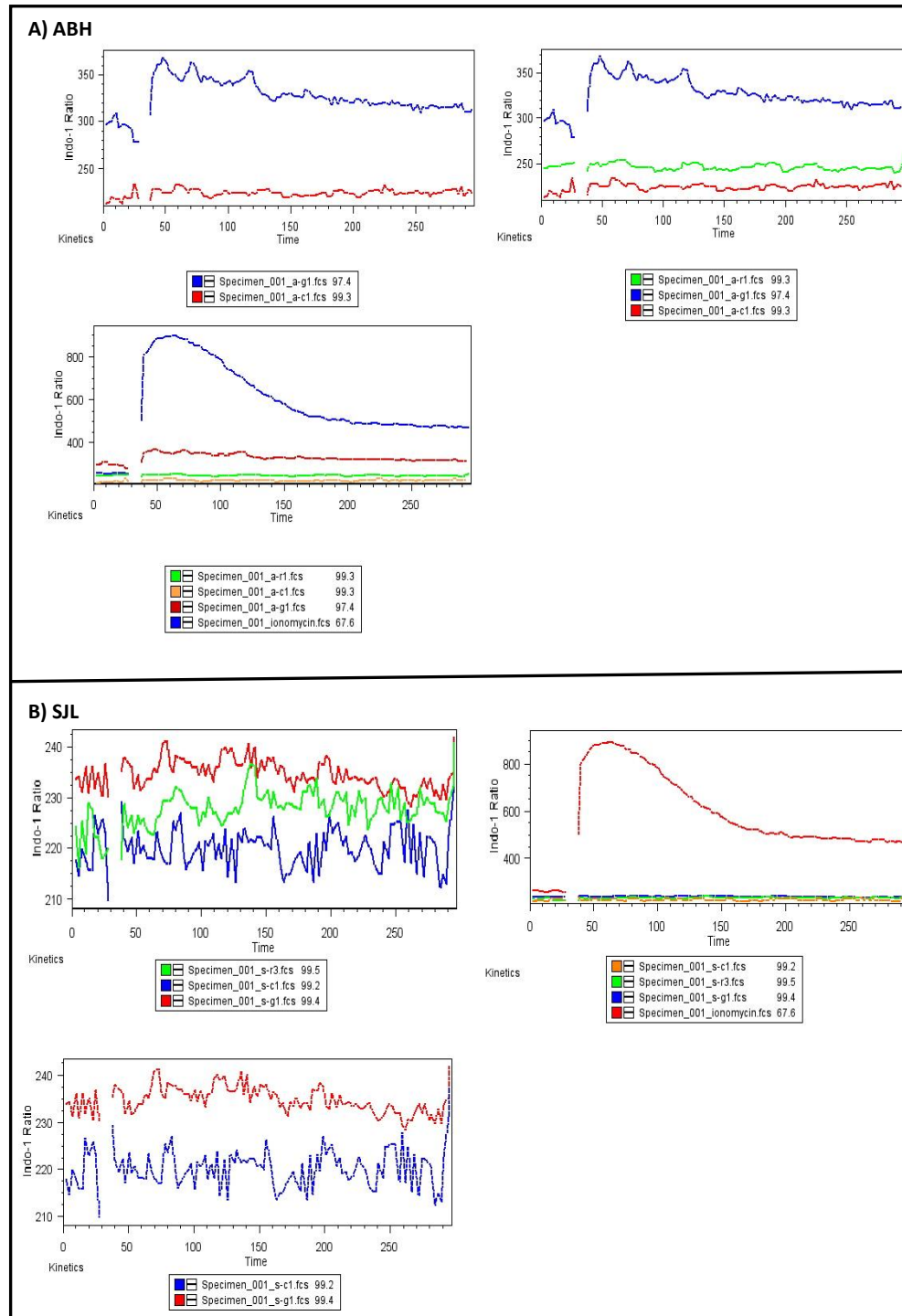


Figure 4.11: Real time intracellular calcium flux in differentiated PC12 cells after addition of sera from ABH and SJL mice immunized with GABHS proteins, RP and CFA. The above images A and B are illustrative of the real time changes in intracellular calcium levels in PC12 neuronal cells after the addition of sera from mice immunized with GABHS proteins,

RP and CFA. The FACS assay involved measurement of Indo-1 (calcium dye) ratio over time. Ionomycin was used as a positive control. A baseline measurement was done for the first 30 s after which the serum sample was added and the changes in intracellular calcium were measured for 5 min. An increase in calcium flux can be observed after the addition of ionomycin and sera samples at 30 s. **(A)** Real time intracellular calcium flux in differentiated PC12 cells after addition of sera from ABH mice immunized with GABHS (α -g1), RP (α -r1) and CFA only (α -c1) at 30 s. **(B)** Real time intracellular calcium flux in differentiated PC12 cells after addition of sera from SJL mice immunized with GABHS (s -g1), RP (s -r3) and CFA only (s -c1) at 30 s.

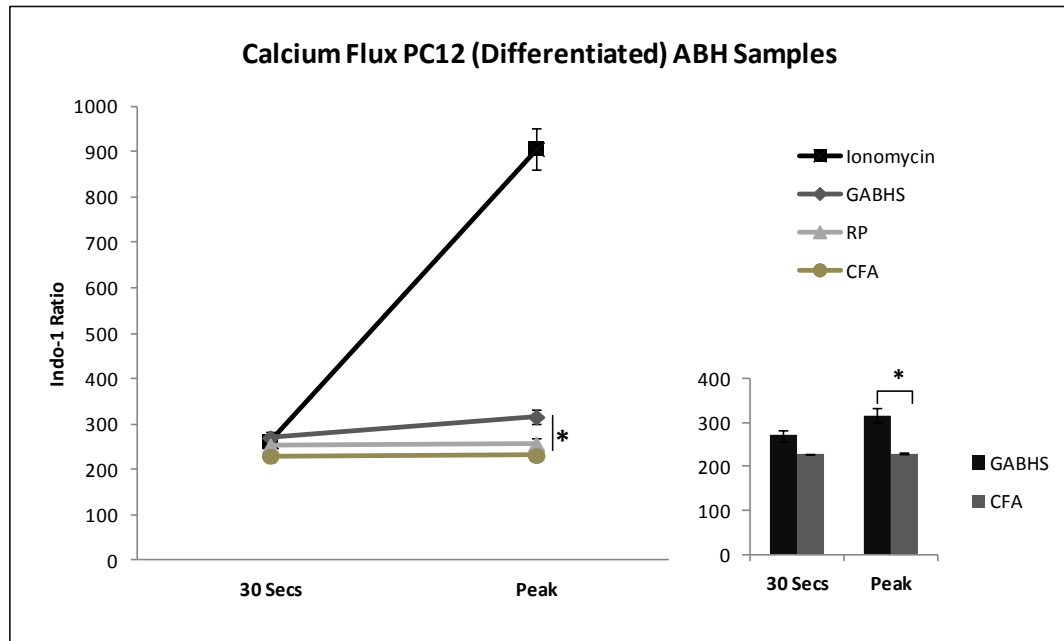


Figure 4.12: Intracellular calcium flux in differentiated PC12 cells following treatment with sera from test and control ABH mice. The graph above depicts the real time intracellular calcium flux as a measure of Indo-1 ratio over time in PC12 neuronal cells after treatment sera from ABH mice immunized with GABHS ($n=3$), RP ($n=3$) and CFA controls ($n=3$). A baseline reading was done for the first 30 s after which the serum sample was added and the changes in calcium flux monitored for 5 min. The Indo-1 ratio was measured before and after the addition of sera samples when it reached its peak. A two-tailed t-test revealed a significant increase in intracellular calcium flux after the addition of patient sera as opposed to healthy controls ($p<0.04$).

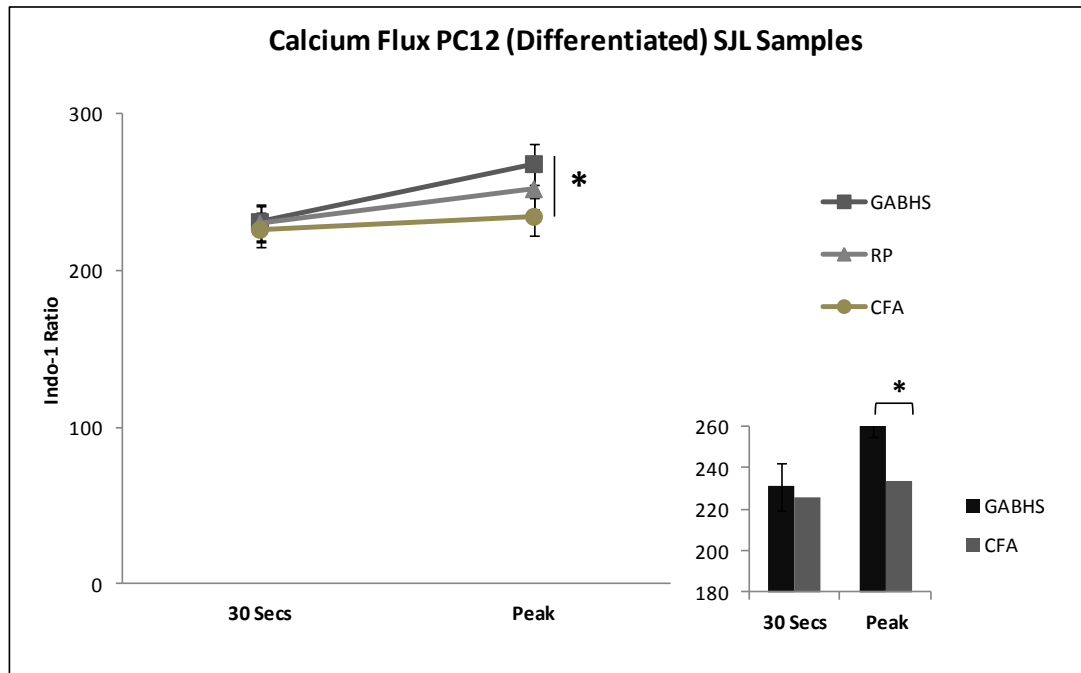


Figure 4.13: Real time intra-cellular calcium flux in PC12 neuronal cells after treatment with test and control sera from SJL mice. The graph above depicts the real time intracellular calcium flux as a measure of Indo-1 ratio over time in PC12 neuronal cells after treatment with sera from SJL mice immunized with GABHS ($n=3$), RPs ($n=3$) and CFA controls ($n=3$). A baseline reading was done for the first 30 s after which the serum sample was added and the changes in calcium flux monitored for 5 min. The Indo-1 ratio was measured before and after the addition of sera samples when it reached its peak. A two-tailed t-test revealed a significant increase in intracellular calcium flux after the addition of patient sera as opposed to healthy controls ($p<0.02$).

4.5 DISCUSSION

It was hypothesized that there was an antibody-mediated autoimmune mechanism as well as bacterial infection contributing to the pathogenesis of neuropsychiatric movement disorders like TS and SC. Apart from clinical evidence and a disease replicating animal model another proof of an autoimmune aetiology comes from demonstrating the autoaggressive action of circulating autoantibodies on living cells. The pathogenic effect of these antibodies was studied in both neuronal and non-neuronal cells in terms of cytotoxicity, apoptosis and intracellular calcium flux.

Using the WST-1 cytotoxicity assay a clear decrease in the number of viable cells was seen in response to patient autoantibodies as compared to those from healthy controls. Similarly a significant increase in apoptosis was observed in both differentiated and undifferentiated cells after treatment with patient antibodies. Patient sera samples were bifurcated into two categories TS and non-TS (SC, EL and dystonia) and both groups were seen to exert the same pathogenic effect on PC12 cells. This finding strengthens the hypothesis that these disorders are likely to be autoantibody mediated and could be part of the same spectrum. A recent study however exhibited that serum autoantibodies only from SC, but not PANDAS or TS patients bound to neuronal surface antigens (Brilot et al., 2011). The cytotoxic and apoptotic effect of patient antibodies was consistent in both undifferentiated and PC12 differentiated into neurons. The main form of enolase dimer on undifferentiated cells is the $\alpha\alpha$ -homodimer (non-neuronal enolase) (Vinores et al., 1981). A change from non-neuronal to neuron specific enolase ($\alpha\gamma$ and $\gamma\gamma$ –dimer) during PC12 differentiation into neurons has also been documented (Schemedel et al., 1980). This confirms that anti-neuronal antibodies found in patients with neuropsychiatric disorders target both α and γ enolase isoforms.

In the present study it was not possible to perform an ATP assay due to technical difficulties. As part of the future studies I will determine the ATP/ADP ratios using a luciferase chemiluminescence assay (Pocock & Nicholls, 1998) to determine whether energy metabolism is altered. ATP levels need to be assessed since apoptosis may be triggered upstream by perturbations in ATP, in particular a fall in ATP. It is also possible that an increase in ATP in some instances given current suggestions that cell surface glycolytic enzymes may actually serve to generate energy at the membrane (Dubinsky et al., 1998). In line with this there is preliminary data using a commercial anti-enolase monoclonal antibody, which causes a marked increase in neuronal ATP but a fall in ADP relative to controls. The time course of such ATP increases needs to be described since an initial stimulation may be followed by a fall, triggering apoptosis, particularly if energy substrate sources are depleted.

There are a number of possible ways in which these autoantibodies can act. Increased signal transduction is one mechanism proposed to alter neuronal cell physiology leading to neurological dysfunction. In 2003, Kirvan et al., demonstrated cross-reactive antibodies in SC that deposit in the basal ganglia and lead to calcium/calmodulin-dependent protein kinases (CaM kinase II) activation in neuronal cells (Kirvan et al., 2003). The CaM kinases are a family of related kinases that are activated in response to increased intracellular calcium concentrations. As seen in my results patient antibodies led to a marked increase in intracellular calcium flux as compared to those from healthy controls. Ca^{2+} influx into cells activates a number of enzymes, including phospholipases, endonucleases, and proteases which lead to damage of structures like DNA and the cytoskeleton (McConkey and Nutt, 2001; Gronski et al., 2009). Although an effect of autoantibodies from patients on calcium flux was demonstrated the nature of the target receptor/channel still needs to be identified which would further help us understand the downstream alterations in signal transduction.

Some initial studies with patient IgG have indicated decreased calcium responses in cultured neurones to a depolarising stimulus. In cultured neurones, enhanced intracellular free calcium provides trophic functions for neuritogenesis and gene transcription, particularly via the NMDA receptor and L-type calcium channel (Helton et al., 2005). The L-type calcium channels preferentially localize to soma and dendrites of neurons (Hell et al., 1993; Simon et al., 2003), but their contribution to action potential-dependent calcium entry in these regions is still unclear. Blocking these pathways with the NMDA receptor antagonist MK801 or the L-type calcium channel antagonist Nifedipine (a dihydropyridine), might trigger apoptosis. In our future experiments The effects of purified monospecific antibodies on KCl-evoked calcium responses will be determined together with NMDA stimulated calcium responses. Single cell calcium (Ca^{2+}) responses can then be measured by fluorescence imaging as previously described (Evans & Pocock, 1999; Pocock and Evans 2000).

Additionally an *in vitro* GABHS infection model possibly should be developed using cultured phagocytes to study dose-dependent apoptosis. Some previous studies of GABHS interactions with epithelial cells assessed apoptosis as an outcome variable. In respiratory epithelial cell lines, internalization of the bacteria was observed to be critical for induction of the apoptotic phenotype (Nakagawa et al., 2001; Timmer et al., 2009). GABHS have been seen to induce apoptosis in human neutrophils (Kobayashi et al., 2003) although the specific virulence factor(s) involved and effects on caspase activation have not yet been explored. Purified SAg and M proteins could be used to study their individual pathogenic effect on cellular and sub-cellular targets.

All functional assays were also carried out using sera from SJL and ABH mice immunized with GABHS proteins, recombinant proteins and CFA. Both SJL and ABH mice are known to

be susceptible to induction of experimental allergic encephalomyelitis (EAE) (Levine and Sowinski, 1973; Goverman and Brabb, 1996). ABH additionally was later found to be highly susceptible to other autoimmune conditions like autoimmune neuritis, autoimmune uveitis, as well as virus-induced demyelination and have therefore been the key strain to study human inflammatory neurological diseases (Amor et al., 2005). High levels of cell cytotoxicity and apoptosis were observed after the addition of sera from mice immunized with GABHS and RP from both strains. However a significant increase in calcium flux was only seen with sera from SJL and ABH immunized with GABHS proteins and not RP. As described previously I could not observe a disease phenotype in these mice due to lack of adequate behavioural testing. At least in an *in vitro* setting it was possible to determine the pathogenic effect of the antibodies raised against the putative autoantigens. The results obtained are promising such that this animal model will be developed further by immunization with individual recombinant proteins to raise mono-specific antibodies.

CHAPTER 5

ENOLASE AS A TARGET AUTOANTIGEN

5.1 INTRODUCTION

5.1.1 Enolase

5.1.1.1 Background

The enzyme enolase also known as phosphopyruvate dehydratase was discovered by Lohmann and Meyerhof in 1934 (Lohman.K 1934). It is responsible for the catalysis of 2-phosphoglycerate (2-PG) to phosphoenolpyruvate (PEP), the penultimate step of glycolysis.

There are three subunits of enolase, α , β , and γ , each encoded by a separate gene that can combine to form five different isoenzymes: $\alpha\alpha$, $\alpha\beta$, $\alpha\gamma$, $\beta\beta$, and $\gamma\gamma$. Alpha-enolase (also called non-neuronal enolase) is ubiquitously expressed in the early stage of embryonic development, beta-enolase is expressed in adult skeletal and cardiac muscles, and gamma-enolase (also called neuron-specific enolase) is expressed in mature neuron and neuroendocrine cells (Marangos and Schmechel, 1987, Deloulme et al., 1997). Using sensitive immunoassays, significant levels of γ enolase have been found in differentiated tissues other than nervous tissues (Haimoto et al., 1985). The structure is highly conserved with a homology of 40-90% between different species (Pancholi, 2001). In mammals there is 82% homology between the amino acid sequences of α -, β -, and γ -enolase monomers (Pancholi, 2001). Studies with discoid lupus erythematosus have demonstrated that the reactive autoepitopes in enolase are also highly conserved from “yeast to man.” This study observed that autoantibodies reacted most strongly with α -enolase but also predicted that γ -enolase would show some reactivity with β -enolase producing the least (Gitlits et al., 1997).

5.1.1.2 Function

Enolase has also been shown to act as a plasminogen receptor on neuronal membranes (Nakajima et al., 1994a) mediating interactions between microglia and dopaminergic neurons (Nakajima et al., 1994b). The enolase binding to plasminogen induces plasminogen transformation into plasmin and is thought to be a virulence factor by preventing the generation of fibrin clots and thus enabling tissue invasion, as described for *S. pyogenes* and *S. Pneumonia* (Fontan et al., 2000). Plasminogen is critical in the host's defence as it dissolves fibrin clots, maintaining homeostasis and vascular potency; however GABHS subverts this fibrinolytic activity, increasing pathogenesis (Pancholi and Fischetti, 1998). Eukaryotic haematopoietic cells – B cells, T cells, neutrophils, and monocytes also express α -enolase on their surface but streptococcal surface enolase SEN has a higher affinity for plasminogen and has been proposed to be the major plasminogen binding protein on the surface of GABHS (Pancholi and Fischetti, 1998; Pancholi, 2001; Witkowska et al., 2005). NSE can form complexes with other glycolytic enzymes (e.g. aldolase C) which monitor oxidative stress levels at the cell surface (Bulliard et al., 1997). In addition, α -enolase when located in the nucleus is a Myc-binding protein (MBP-1) playing a vital and crucial role in cell growth and differentiation (Terrier et al., 2007). In addition, heat shock protein, Myc-binding protein, eye τ -crystallin protein, and an endothelial stress protein (Pancholi, 2001).

Proteins that are structurally similar to human enolase have also been observed on the surface of other bacteria including *Streptococcus pneumoniae*, *Escherichia coli*, *Klebsiella pneumoniae*, *Hafnia*, *Citrobacter*, *Enterobacteriaceae* and *Pseudomonas aeruginosa* (Witkowska et al., 2005). In *E.coli*, enolase is an integral component of the RNA degradosome (Kuhnel and Luisi, 2001; Carpousis, 2002). Its role has been hypothesized to direct subcellular compartmentalization of degradosome to the membrane (Liou et al. 2001;

Morita et al. 2004). These enolase-like proteins have been shown to react with rabbit antibodies specific for human β -enolase and have been observed in patients with Buerger disease and atherosclerosis (Witkowska et al., 2005). The aetiology of these conditions is unknown but previous studies have suggested a bacterial cause (Witkowska et al., 2005). What is also unclear is exactly how α -enolase and enolase-like proteins are present on the surface of these cells. These proteins have no signal sequence or hydrophobic domain to translocate the protein from the cytoplasm to the cell membrane (Pancholi, 2001). It has therefore been suggested that enolase and other proteins with these characteristics should be classed as a special variety of surface protein (Pancholi, 2001).

5.1.1.3 Associated Disorders

Studies have reported the presence of enolase-specific autoantibodies in a range of psychiatric, degenerative and inflammatory disorders, including discoid lupus erythematosus (Terrier et al., 2007), autoimmune polyglandular syndrome type 1 primary, and secondary membranous nephropathy, cancer-associated retinopathy, primary biliary cirrhosis (Akisawa et al., 1997), mixed cryoglobulinemia with renal involvement, cystoid macular edema, endometriosis (Gitlits et al., 2001). In addition, autoantibodies which cross react with GABHS and human α -enolase have been observed in ARF patients (Fontan et al., 2000). ARF is one of the most important sequelae of upper respiratory tract infection occurring due to GABHS (McDonald et al., 2004).

Antibodies against glycolytic enzymes have also been observed in optic neuritis. Out of 209 samples tested for anti-optic nerve autoantibodies, 55% showed specific neuronal autoantibodies. Autoantibodies specific to classical glycolytic enzymes involved in energy production (α and γ enolases, glyceraldehyde 3-phosphate dehydrogenase) also reacted with retinal antigens (Adamus et al., 2011).

In a recent study ninety-six participants with OCD were tested for the presence of anti-streptolysin-O titres (ASOT) and the presence ABGA. The findings were compared with those in a control group of individuals with depression (n= 33) and schizophrenia (n= 17). ABGA were tested for with western blots using three recombinant antigens; aldolase C, enolase and pyruvate kinase. Positivity for ABGA was observed in 19/96 (19.8%) participants with OCD compared with 2/50 (4%) of controls (Fisher's exact test $P = 0.012$). The majority of positive OCD sera (13/19) had antibodies against the enolase antigen (Nicholson et al., 2012).

5.1.2 Sodium Potassium ATPase (Na^+/K^+ -ATPase)

5.1.1.2 Background

Sodium Potassium ATP-ase (Na^+/K^+ -ATPase) is a heterodimeric integral membrane protein responsible for carrying out coupled extrusion and uptake of Na^+ and K^+ ions across the plasma membrane of cells (Kaplan JH, 2002). The Na^+/K^+ -ATPase is composed of a 100 kDa α -subunit with ten transmembrane segments and a glycosylated β -subunit of about 55 kDa. Four distinct α -subunits and the β subunit isoforms have been identified, and there appears to be no preferential association of particular subunits with each other (Peng et al., 1997; Mao et al., 2005).

The pump is composed of multiple isoforms and the isoform distribution varies with the tissue and during development (Kaplan, 2002). The alpha 1 isoform is the major isoform in the kidney and the alpha2 isoform is the predominant one in skeletal muscle (Lingrel et al. 2007). While all three isoforms are found in the brain, the alpha 3 isoform is located essentially in neurons while the alpha 2 isoform is found in astrocytes and some limited neuronal populations. Interestingly the alpha 4 isoform is found exclusively in the mid region of the sperm tail (Lingrel et al. 2007).

5.1.2.2 Function

The pump plays a central role in a variety of physiological processes like differentiation (Smith et al., 1982), proliferation (Rozengurt and Heppel, 1975), propagation of action potential of nerves and muscles (Thomas E, 1972) and regulation of cellular volume (Rolfe and Brown, 1997). It also functions as signal transducer/integrator to regulate the mitogen-activated protein kinase pathway (MAPK), mitochondrial reactive oxygen species (ROS) production, as well as intracellular calcium (Liu et al., 2000; Aizman and Aperia, 2003). In neurons, the Na^+/K^+ -ATPase can account for up to two-thirds of the cell's energy expenditure (Howarth et al., 2012).

Lipid rafts are cholesterol- and shingolipid-enriched membrane microdomains implicated in membrane signalling and trafficking (Calder and Yaqoob, 2007). These specialized microdomains serve as organizing centres and compartmentalize cellular processes like the assembly of signalling molecules, regulating neurotransmission and receptor trafficking and membrane protein trafficking (Thomas et al., 2004; Calder and Yaqoob, 2007). Although recent reports have begun to describe molecules associated with rafts, their protein composition remains largely unknown, especially in neuronal cells (Ledesma et al., 2003). Proteomic analysis of the low-density fraction of lipid rafts revealed the α -subunit of Na^+/K^+ -ATPase as the protein with the highest significance score (Welker et al., 2007). Na^+/K^+ -ATPase forms a signalling complex composed of multiple receptors (EGFR, IP3R), structural proteins (ankyrin, caveolin) and protein and lipid kinases (Src-kinase, PI3K) (Brady et al., 2011)

Enolase being a glycolytic enzyme, as expected, is found in the cytosol. However as mentioned above it is also located on the neuronal surface, where it serves alternate functions like acting as a receptor for plasmin/plasminogen and has also been shown to be

a trophic factor for dopaminergic neurons (Liu et al., 2007; Plow and Das, 2009). Enolase along with the other glycolytic enzymes might be positioned in the lipid rafts and may interact with other membrane proteins, e.g. ion channels like the Na^+/K^+ -ATPase. Bi-dimensional gel analysis and pharmacological raft lipid manipulation of purified detergent-insoluble raft fractions from primary cultures of hippocampal neurons have led to the identification of neuronal raft proteins. Enolases were found amongst these proteins and functional studies demonstrate their participation in plasminogen binding (Ledesma et al., 2003; Camoletto et al., 2009). The rafts were found enriched with several other plasminogen binding molecules and the exclusive activation of plasminogen to the protease plasmin in these microdomains (Ledesma et al., 2003). These observations indicate that neuronal rafts may not only play a role in intracellular signalling but might also be involved in extracellular/membrane protein proteolysis (Ledesma et al., 2003). The active extrusion of Na^+ ions from human erythrocytes is inhibited by low concentrations of fluoride ion (Maizels, 1951). This has been attributed to glycolytic inhibition caused by fluoride ion (Embden and Haymann, 1924), which leads to a decline in cellular concentration of adenosine triphosphate (ATP), the presumed energy source for the Na^+ pump (Gardos and Straub, 1957; Eckel, 1958; Millmann and Omachi, 1972).

5.2 HYPOTHESIS AND AIMS

The membrane glycolytic enzymes are closely involved in the energy provision and maintenance of ion channels on the neuronal membrane, trophic support and other functions. Based on previous findings that have identified enolase as a potential autoantigen in the present study it was hypothesized that antibodies from patients with neuropsychiatric movement disorders like TS, SC and EL will inhibit the enzymatic activity of this glycolytic enzyme.

In addition the hypothesis that all ion channel proteins exist in the plasma membrane in a regulatory complex with one or more proteins and enolase might be part of that complex that participate in the modulation of channel activity has been pursued. Hence inhibition of enolase activity might be resulting in dysregulation of Na^+/K^+ -ATPase thereby leading to disruption of normal cellular processes.

The main aim of this study was analyse the effect of autoantibodies from patients diagnosed with postulated post-streptococcal disorders neuropsychiatric disorders like TS, EL and SC on the enzymatic activity of cell surface enolase using PC12 neuronal cells. To study the expression of Na^+/K^+ -ATPase and NSE in PC12 neuronal cells and see if they are co-expressed using fluorescence confocal microscopy.

5.3 MATERIALS AND METHODS

5.3.1 Sera Samples

A total of 49 patient sera samples were used for the enolase activity assay. The cohort included sera samples from patients diagnosed with TS (n=22) and other disorders including SC, EL and dystonia (n=27). The TS patient sera samples were kindly provided by Dr. Davide Martino, University of Bari, Italy. Sera samples from patients with SC, EL and dystonia were provided by Dr. Andrew Church, Institute of Neurology, London. Furthermore sera from healthy controls (n=20) kindly provided by Dr. Ruth Dobson, Department of Neuroscience and Trauma, Queen Mary University of London were also tested in the assay. Patient material was obtained with informed consent and appropriate ethical review.

5.3.2 PC12 cells

PC12 is a cell line derived from a pheochromocytoma (neuroendocrine tumour) of the rat adrenal medulla (Greene and Tischler, 1976). When exposed to neurotrophins like the nerve growth factor (NGF) neoplastic PC12 cells undergo a series of biochemical and morphological changes thereby differentiating into neural cells (R.J.Walters, 2002).

PC12 cells grown with or without nerve growth factor (NGF) contain dense core chromaffin-like granules up to 350 nm in diameter. The NGF-treated cells also contain small vesicles which accumulate in process varicosities and endings. PC12 cells synthesize and store the catecholamine neurotransmitters dopamine and norepinephrine (Cunningham et al., 2001).

PC12 cells were cultured in T75 culture flasks coated with Collagen I, Rat Tail (Invitrogen Life Technologies, UK). The cells were maintained in RPMI 1640 medium (Sigma-Aldrich, UK) supplemented with 50mL horse serum (Lonza 14-403F), 25mL foetal calf serum (Sigma-Aldrich, Poole, UK), 5mL penicillin/streptomycin (Sigma-Aldrich, UK) and 5mL Non-Essential amino acid solution (Sigma-Aldrich, UK) in a humidified incubator at 37°C and 4.5% CO₂. The cells were passaged every 2-3 days in a ratio of 1:5. To induce differentiation into neurons the cells were treated with 50 ng/mL of NGF (Invitrogen Life Technologies, UK) every 2 days for upto a week.

5.3.3 Cell Fractionation

PC12 cell fractions were prepared using the complete cell fractionation kit (Promokine, Germany) to carry out enolase activity assay on whole cells and fractions. PC12 cells (4-8 x 10⁶) were collected by centrifugation at 700 x g for 5 minutes. The cells were then washed with 5-10mL of ice-cold PBS (Invitrogen Life Technologies, UK) and centrifuged at 700 x g for 5 mins. The cell pellet was then resuspended in 1mL of ice-cold PBS and transferred to an eppendorf tube. A spin for 5 min at 700 x g was done and the supernatant was removed.

The pellet was then resuspended in 400 μ L of Cytosol Extraction Buffer-Mix (CEB-mix containing dithiotheitol (DTT) and protease inhibitor cocktail) and incubated on ice for 20 min with gentle tapping (3-4 times) every 5 min. Once again the cell samples were centrifuged at 700 x g for 10 min and the supernatant was collected (**Cytosolic Fraction**).

For the membrane fraction the pellet was re-suspended in 400 μ L of ice-cold Membrane Extraction Buffer-A Mix (MEB-A Mix containing DTT and protease inhibitor cocktail) to which 22 μ L of Membrane Extraction Buffer-B, was added. The samples were vortexed for 5 seconds and incubated on ice for 1 min before centrifugation for 5 min at 1000 x g. The supernatant was immediately transferred to a clean pre-chilled tube (**Membrane/Particulate Fraction**).

5.3.4 Enolase activity assay

Enolase isoforms and their dimers are some of the known target of anti-neuronal antibodies (Dale et al., 2004). An assay for enolase was carried out on whole adhered growing cells to determine if the activity of enolase is affected by though binding of anti-neuronal antibodies. It is more likely that antibodies bind to targets on cell surface, therefore, the cells were not permeabilized when measuring the enzymatic activity of enolase. However analysis of the enolase activity was performed on whole PC12 neuronal cells and various cell fractions including membrane and cytosol to identify the targets of these autoantibodies.

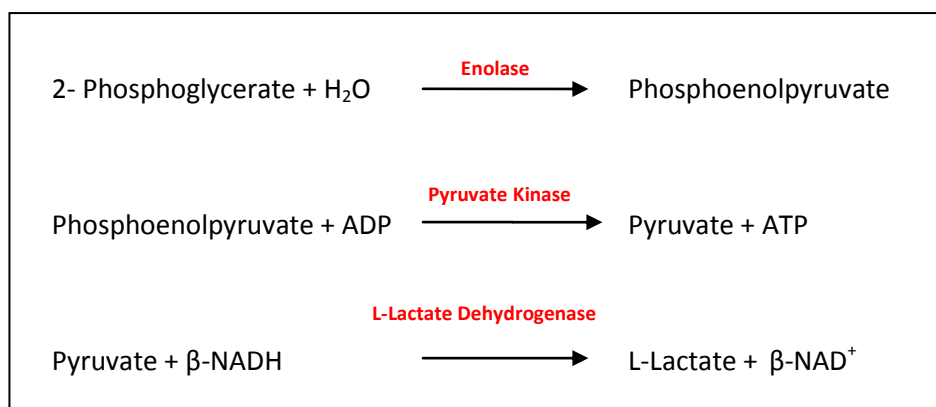


Figure 5.1: Enzymatic reaction in the enolase assay. Enolase converts 2-Phosphoglycerate (DPG) to phosphoenolpyruvate (PEP), which is further converted to pyruvate through pyruvate kinase (PK). The enzyme lactate dehydrogenase (LDH) converts pyruvate to lactate by oxidation of NADH. Enolase activity is revealed by the decrease in NADH that can be measured through changes in absorbance at 340 nm.

PC12 cells were cultured onto rat tail, collagen I (Invitrogen life technologies, UK) coated flat bottomed 96 well cell culture plates. The cells were differentiated into neurons by treatment with NGF (50 ng/mL) every alternate day over a period of 7 days. After the cells had fully differentiated into neurons 1:100 diluted serum samples from patients with TS, EL, SC and dystonia along with healthy controls were added to the cells which were then incubated with the antibodies for 24 hs. Two positive controls were used in this assay- 1:100 diluted monoclonal antibody (mAb) against neuron specific enolase (NSE) (1 mg/mL) (Abcam, UK) and 0.5 mM sodium fluoride (Abcam, UK). Fluoride inhibits the activity of enolase by binding Mg^{2+} thereby hindering interactions between Mg^{2+} and the substrate 2-PG (Hoorn et al., 1974).

50 μL reaction mixture containing 4mM 2-phosphoglycerate barium salt, 1mM β -nicotinamide (NADH), 50mM magnesium sulphate (MgSO_4), 200mM potassium chloride (KCl), 2.6mM adenosine di-phosphate (ADP), 0.3 unit of lactate dehydrogenase (LDH) and

0.2 units of pyruvate kinase (PK) dissolved in medium was added and the plate which was incubated at 37°C. Absorbance was measured at 340 nm every 15 min for up to 1 h 30 min. The data plotted shows reading upto an hour as no significant changes in absorbance were observed after that. The absorbance measured was directly proportional to the levels of NADH which in turn was inversely proportional to enolase activity. Hence higher the absorbance, lower the enolase activity.

5.3.5 Immunocytochemistry (ICC)

PC12 cells were grown and differentiated on coverslips coated with type I rat tail collagen as described above. Once fully differentiated into neuronal cells they were fixed in 4% paraformaldehyde (PFA) pH7.4 for 15 min at RT followed by washes with ice cold PBS. The samples were then blocked with 1% BSA in PBS containing 0.1% tween for 30-60 min to block unspecific binding of the antibodies followed by incubation in the diluted primary antibodies prepared in the blocking solution at a dilution of 1:500 for 1 h at RT or overnight at 4°C. Three primary antibodies were used in the study as listed in the table below: anti-neuron specific enolase (NSE) (Abcam, UK), anti-sodium potassium ATPase (Na^+/K^+ ATPase) (Abcam, UK) and anti-neurofilament (NFL) (Abcam, UK). The antibody solution was then decanted and the cells were washed 3X PBS, 5 min per wash. The cells were then incubated with the appropriate secondary antibody prepared in 1% BSA for 1 h at RT in dark. Secondary antibody solution was then decanted and the cells were washed three times with PBS for 5 min each in the dark. The cells were then incubated with 0.1-1 µg/mL Hoechst or DAPI (DNA stain) for 1 min and rinsed with PBS. The coverslips were mounted onto superfrost slides (VWR, UK) with a drop of DEPEX mounting medium and sealed with nail varnish to prevent drying and movement under microscope. The slides were then stored at +4°C. The slides were viewed using the Zeiss LSM 510s laser scanning confocal microscope.

Primary Antibody	Secondary antibody	Product Source
Mouse monoclonal to neuron specific enolase (NSE)	Fluorescein isothiocyanate (FITC) conjugated goat anti-mouse (Green)	Abcam, UK
Rabbit polyclonal to sodium potassium ATPase (Na^+/K^+ ATPase)	Tetramethylrhodamine-5-(and 6)-isothiocyanate (TRITC) conjugated sheep anti-rabbit (Red)	Abcam, UK
Chicken polyclonal to 68kDa neurofilament	DyLight™ 405-Conjugated donkey anti-chicken (Blue)	Abcam, UK Jackson ImmunoResearch Laboratory, USA

Table 5.1: Table summarising the primary and secondary antibodies used for staining PC12 neuronal cells. The antibodies listed above were used to study the expression of neuron specific enolase (NSE), sodium potassium ATPase (Na^+/K^+ -ATPase) and neurofilament (NFL) in PC12 neuronal cells using confocal microscopy.

5.4 RESULTS

5.4.1 Enolase Activity Assay

Enolase is one of the autoantigen implicated in postulated post-streptococcal disorders mentioned above. Enolase activity was measured in PC12 neuronal cells: whole cells, cytosol and membrane fraction after the addition of sera samples from patients diagnosed with TS and EL (n=6) and healthy controls (n=5). The assay was carried out on both membrane and cytosol fractions to investigate where the antibody binding occurs. The enzymatic assay entailed measurement of NADH which bears an inverse relation with enolase activity i.e., the higher the NADH the lower the enolase activity and *vice versa*. The production of NADH concentration was measured at an absorbance of 340 nm. From the graphs below it can be concluded that sera antibodies bind to enolase present on the cell membrane and inhibit its activity. The statistical analysis shows a significant inhibition of enolase activity in both PC12 whole neuronal cells ($p<0.04$) and its membrane fraction ($p<0.001$) after the addition of sera from patients as compared to healthy controls.

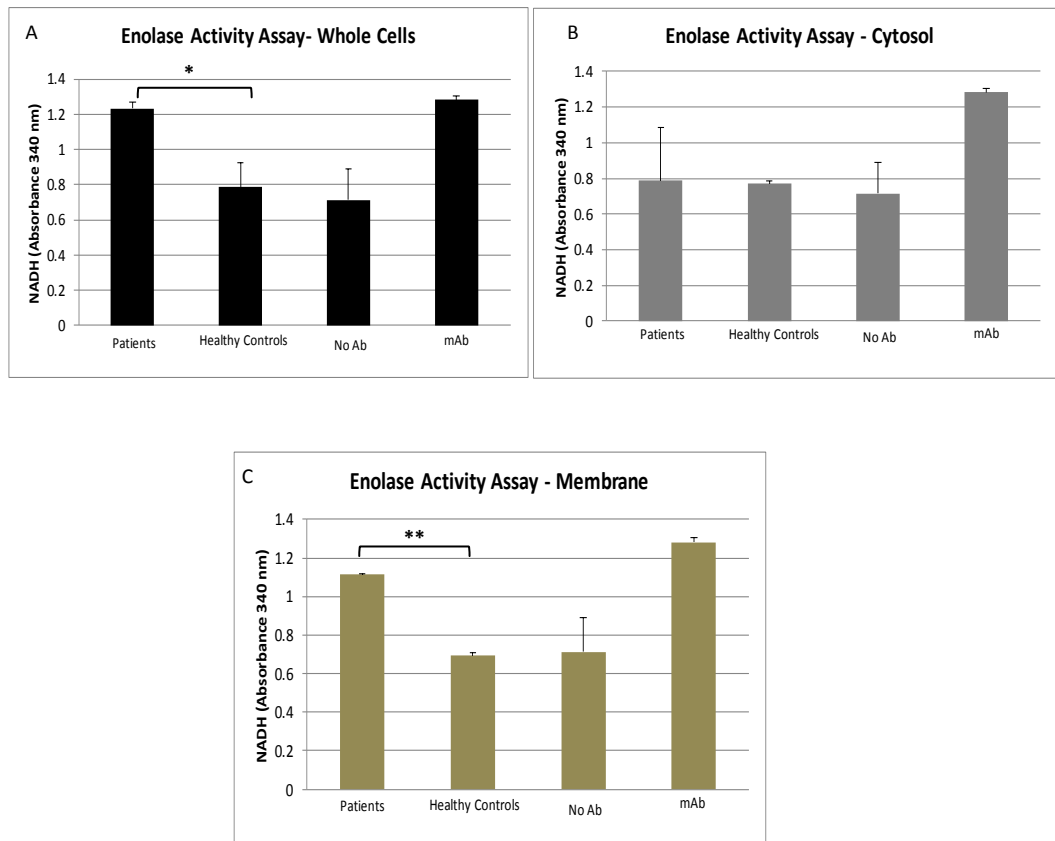


Figure 5.2: Enolase activity assay conducted on PC12 neuronal cell fractions. Graphs A, B & C above depict the changes in levels of NADH (absorbance @340 nm) measured over a period of 1 hour in PC12 neuronal whole cells and fractions (membrane and cytosol) after treatment with sera samples from patients diagnosed with TS and EL as compared to healthy controls. Levels of NADH bear an inverse relation to levels of enolase activity i.e., higher the NADH lower the enolase activity and vice versa. Both mAb against NSE and sodium fluoride were used as positive controls. **(A)** Shows the levels of NADH in PC12 neuronal cells after the addition of sera from patients with TS and EL versus healthy controls. A two- tailed T-test revealed a significant inhibition of enolase activity after the addition of patient sera as compared to control ($p<0.04$). **(B)** Shows the levels of NADH in the cytosolic fraction of PC12 neuronal cells after the addition of sera from patients with TS and EL versus healthy controls. No significant difference in enolase activity was observed between the patients and controls. **(C)** Shows the levels of NADH in the membrane of PC12

neuronal cells after the addition of sera from patients with TS and EL versus healthy controls. A two- tailed t-test revealed a significant inhibition of enolase activity after the addition of patient sera as compared to control ($p<0.001$).

Once determined that antibodies target the enolase present on the plasma membrane sera samples from additional samples from patients diagnosed with TS, EL, SC and dystonia along with healthy controls were tested for their action on the enzymatic activity of enolase in non-permeabilized PC12 neuronal cells. Statistical tests show a significant decrease in enolase activity after the addition of sera from patients as compared to controls. Two- tailed t-test comparing patients and controls at each time point confirmed a significant inhibition of enolase activity of patient sera as compared to control ($p<0.0001$).

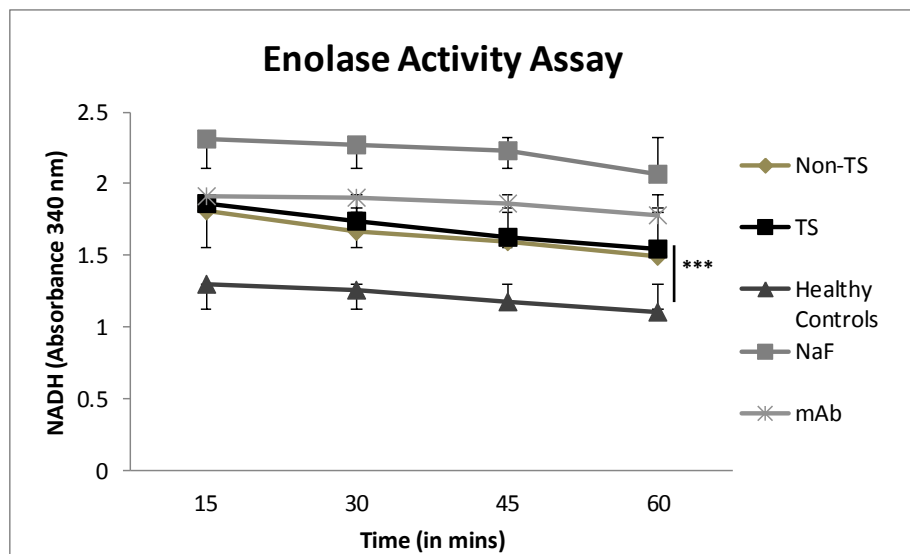


Figure 5.3: Enolase activity assay conducted on PC12 neuronal cells using patient and control sera. The graph above shows the changes in levels of NADH (absorbance @340 nm) measured over a period of 1 hour in PC12 neuronal cells after treatment with sera samples from patients diagnosed with TS, Non-TS disorders (SC, dystonia and EL) and healthy controls. Level of NADH is inversely proportional to enolase activity i.e., higher the NADH lower the enolase activity and vice versa. Sodium fluoride and mAb against neuron-specific

enolase were used as positive controls. Statistical analysis revealed increased inhibition of enolase activity (higher NADH) in PC12 neuronal cells at every time point (15, 30, 45 and 60 min) after the addition of patient sera as compared to controls. Two- tailed T-tests comparing patients and controls at each time point (15, 30, 45 and 60 min) yielded significantly stronger inhibition of patient sera compared to control sera at each of the time points explored ($p < 0.0001$).

5.4.2 Co-expression Na^+/K^+ -ATPase – Neuron specific enolase

The expression of Na^+/K^+ -ATPase and NSE in PC12 cells was studied both before and after differentiation into neurons using fluorescent and confocal microscopy was examined. Undifferentiated cells were counterstained with DAPI that stains the nuclei while for differentiated cells a third antibody against NFL was used which serves as a neuronal marker. 40x images of undifferentiated PC12 cells show the co-expression of Na^+/K^+ -ATPase (red), NSE (green). Although the staining seems diffused the expression of the both Na^+/K^+ -ATPase and NSE can be seen in the plasma membrane of the cells. 100x images of PC12 neuronal cells stained with show similar co-expression of Na^+/K^+ -ATPase (red), NSE (green) and NFL (blue).

5.4.2.1 Undifferentiated PC12 Cells

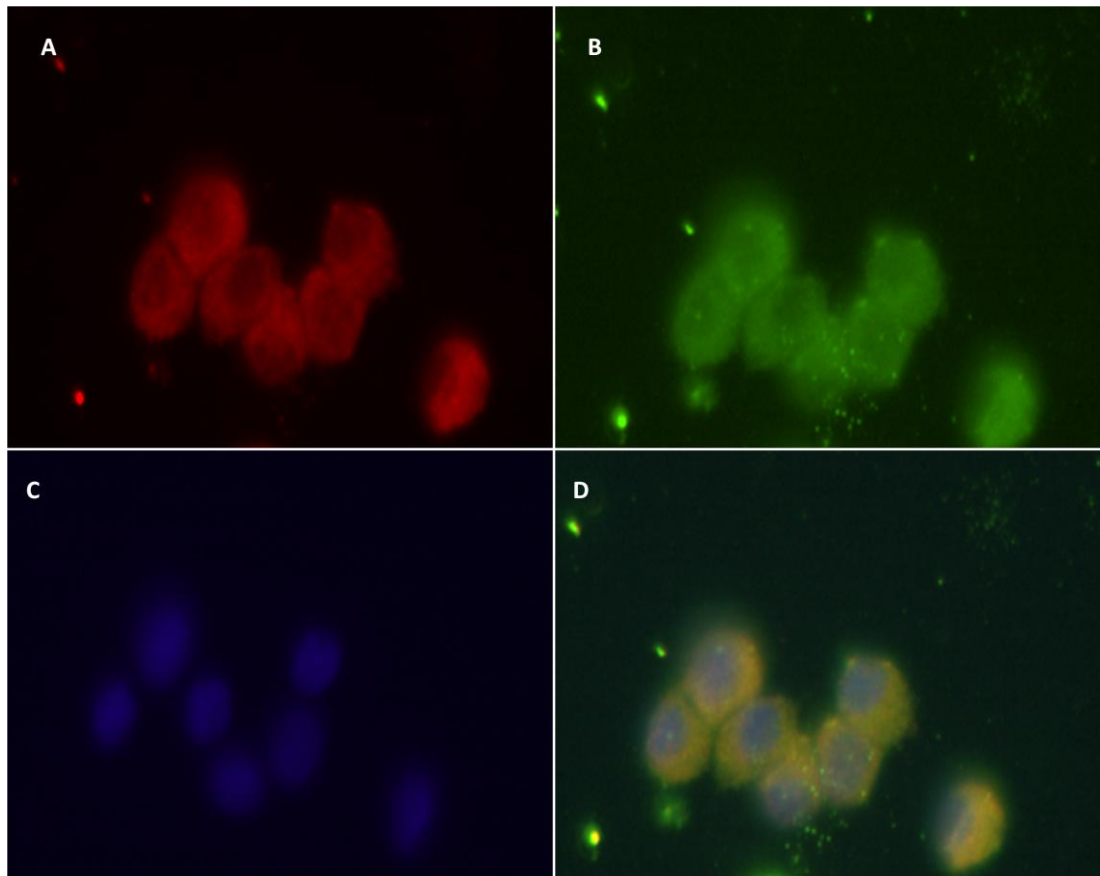


Figure 5.4: Fluorescent images of undifferentiated PC12 cells stained for Na⁺/K⁺-ATPase and NSE. 40X images A to D of PC12 undifferentiated stained with fluorescent tagged antibodies against Na⁺/K⁺-ATPase and NSE and counterstained with DAPI. **(A)** TRITC conjugated Na⁺/K⁺-ATPase. **(B)** FITC conjugated mAb against NSE. **(C)** DAPI stained nuclei. From the staining pattern above both Na⁺/K⁺-ATPase and NSE appear to be co-expressed. Although the staining seems diffused they are expressed primarily in the plasma membrane of undifferentiated PC12 cells.

5.4.2.2 Differentiated PC12 Cells

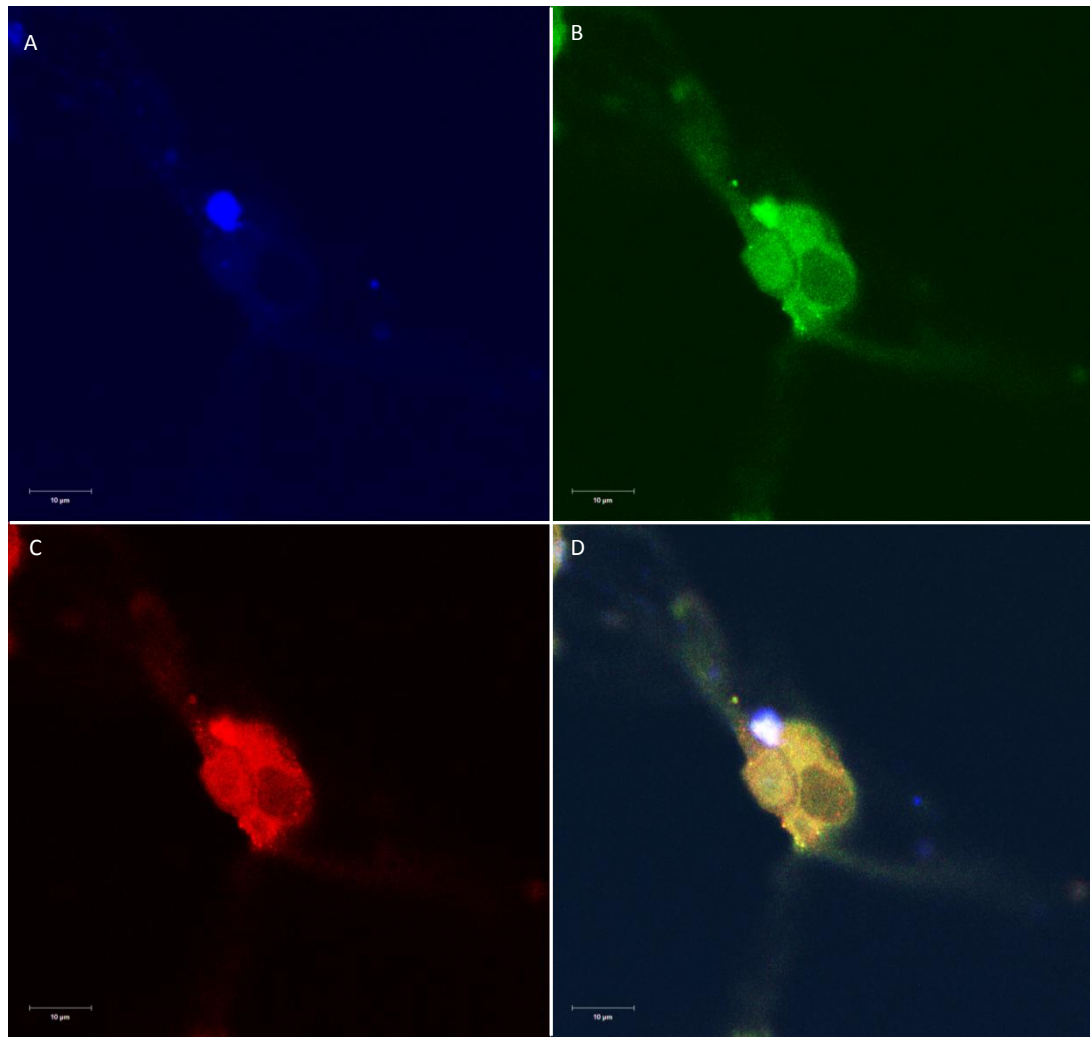


Figure 5.5: Confocal images of differentiated PC12 cells stained for NFL, NSE and Na^+/K^+ -ATPase. 100X confocal images A to D above show PC12 cells differentiated into neurons stained with antibodies against NFL, NSE and Na^+/K^+ -ATPase respectively. **(A)** DyLight™ 405 conjugated antibody against 68kDa NFL. **(B)** FITC conjugated mAb against NSE. **(C)** TRITC labelled polyclonal antibody against Na^+/K^+ -ATPase. **(D)** An overlay of images A, B and C. From the staining pattern observed above Na^+/K^+ -ATPase and NSE seem to be co-expressed in PC12 neuronal cells. Once again the expression seems to be primarily in the membrane of the neuronal cells.

5.5 DISCUSSION

In the present study the role of enolase as a target autoantigen in postulated post-streptococcal CNS disorders was investigated further. Enolase isoforms α and γ and their dimers are some of the known targets of anti-neuronal antibodies (Dale et al., 2004; 2006). Enolase has been implicated as a target autoantigen in a range of non- CNS autoimmune disorders. Autoantibodies directed against a pituitary cytosolic protein identified as α -enolase were detected in 70% of patients with biopsy- proven lymphocytic hypophysitis compared with 9.8% of normal subjects (Crock, 1998; O'Dwyer et al., 2002). Antibodies to alpha enolase have also been found in sera from patients with SLE, particularly those with renal disease (Moodie et al., 1993). An evaluation of antibody titers from patients with ARF has shown the serum to have higher levels of antibodies that react with the human and bacterial enolases compared to serum samples from patients with streptococcal pharyngitis or healthy control subjects (Fontan et al., 2000).

As a glycolytic enzyme enolase is found in the cytoplasm, but it is also found on the cell surface especially on the synaptic membrane (Ueta et al., 2004). The function of enolase on the synaptic membrane is not fully understood but it is known to possess enzymatic activity (Pancholi V., 2001). Fluorocytometric analysis has also revealed that anti-streptococcal enolase antibodies react with the enolase expressed on the surface of haematopoietic cells (Fontan et al., 2000). There is only a poor influx of the enolase educt 2-phosphoglycerate (DPG) into the cells (Hamasaki et al., 1978) so that the main enolase activity measured in this assay comes from the surface enolase and released enolase. So in this study it was attempted to determine if the activity of cell surface enolase present in PC12 neuronal cells was directly affected though binding of anti-neuronal antibodies present in sera of patients diagnosed with TS, SC, EL and dystonia. This method differed from the assay protocols previously described which measured the transformation of NADH to NAD after the

addition of a test solution containing enolase (Pancholi and Fischetti, 1998; Whiting et al., 2002).

A marked inhibition of the enzymatic activity of enolase was after the addition of sera antibodies from patients as opposed to healthy controls. This confirms cell surface enolase as a target autoantigen in this group of neuropsychiatric movement disorders. Future work revolves around further refining the enolase enzymatic assay and defining the threshold values for both patient and control populations such that it can be introduced as a diagnostic test to detect the presence of anti-enolase antibodies.

The $\alpha 1$ subunit of Na^+/K^+ -ATPase contains multiple structural motifs that interact with soluble, membrane and structural proteins including Src, caveolin-1, phospholipase C- γ , PI3 kinase, IP3 receptor, BiP, calnexin, cofilin, and ankyrin (Barwe et al., 2005). Binding to these proteins not only regulates the ion-pumping function of the enzyme, but it also conveys signal-transducing functions to the Na^+/K^+ -ATPase (Kaplan, 2005; Li and Xie, 2009). As seen in the previous chapter anti-neuronal antibodies present in sera from patients diagnosed with TS and other postulated post-streptococcal antibodies result in cell cytotoxicity, apoptosis and increased calcium flux. Also in the present chapter enolase has been demonstrated as one of the targets for these antibodies. I postulated that enolase might be one of the proteins associated with the Na^+/K^+ -ATPase that regulates numerous cell functions in various types of organs and cells including cell motility, cell proliferation, glycogen synthesis, apoptosis, hypertension, intracellular calcium signalling, cardiac hypertrophy, renal and cardiac remodelling, epithelial cell tight junction, vascular tone homeostasis, and sodium homeostasis (Liu et al., 2012). Following this hypothesis undifferentiated and PC12 cells differentiated into neurons were stained for enolase and Na^+/K^+ -ATPase and found the two to be co-expressed. Diffused staining was repeatedly

observed despite using various methods of cell fixation and blocking. Hence this experiment needs to be optimised with a further set of antibodies and other neuronal cell lines.

As mentioned earlier it was postulated that enolase and Na^+/K^+ -ATPase might be present on lipid rafts that are glycolipoprotein microdomains present in the plasma membrane of the cell. The size of the lipid rafts are ranges from 10–200 nm (Pike, 2008). Their size being below the classical diffraction limit of a light microscope has made it difficult to visualize them. Future experiments would involve staining the rafts with fluorophores conjugated to cholera-toxin B-subunit, which binds to the raft constituent ganglioside GM1.

CHAPTER 6

NMDAR AND VGKC ASSAY

6.1 INTRODUCTION

6.1.1 N-Methyl-D-Aspartate Receptor (NMDAR)

6.1.1.1 Background

L-Glutamate is a major excitatory neurotransmitter in the mammalian CNS acting via two classes of receptors, ligand gated ion channels (ionotropic) and G-protein coupled (metabotropic) receptors (Dongen, 2009). The *N*-methyl-D-aspartate receptor (NMDAR) is a specific type of ionotropic receptor belonging to the glutamate receptor superfamily together with AMPA (α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) and kainate receptors (Dingledine et al., 1999). Both NMDA and non-NMDA receptors are activated by the endogenous transmitter, L-glutamate, however, L-aspartate, a putative endogenous transmitter appears to activate NMDA only (Patneau and Mayer, 1990). Glycine, which was first reported to potentiate NMDAR activation at submicromolar levels (Johnson and Ascher, 1987), was later shown to be an essential co-agonist for this receptor (Kleckner and Dingledine, 1988). NMDA receptors are inactive at resting membrane potential this is due to a voltage dependent block of the pore by magnesium ions (Mayer et al., 1984). Their activation is secondary to kainate or AMPA receptor activation that leads to depolarization of the neuron causing the release of the Mg^{2+} blockade (Ascher and Nowak, 2009). The NMDAR is most abundant in the cortex, basal ganglia, and sensory pathways of the nervous system, but have also been identified in a variety of non-neuronal and peripheral locations (Bolton and Paul, 2006).

Electrophysiological studies using recombinant receptors suggest that functional NMDA receptors consist of heteromers containing combinations of two subunits (1) NR1, which is

essential for channel activity (2) NR2 which modulates the properties of the channels (Dunah et al., 1999). Individual subunits and multiple receptor isoforms that arise by selective splicing of the NR1 transcripts and differential expression of the NR2 subunits have been well characterized and found to have distinct brain distributions and diverse functional properties (Flores Soto et al., 2012). The binding sites for the two NMDA agonists, glutamate and glycine are found on different subunits - glycine binds to the NR1 subunit while glutamate binds to the NR2 subunit. Both binding sites interact allosterically and this is one of the reasons why both subunit types are required to generate a fully functioning receptor (Lester et al., 1993). A related gene family of NR3 subunits is the most recently discovered members of the NMDAR family. They form excitatory glycine receptors when co-assembled with NR1 that are calcium-impermeable and unresponsive to glutamate (Chatterton et al., 2002). The lack of agonists or antagonists selective for a given subunit of NMDA receptors has made it difficult to understand the subunit expression, composition, and post-translational modification mechanisms.

The NMDAR is highly permeable to calcium ions and plays a key role in the plasticity of synapses, which is believed to underlie memory and learning, as well as the development of the nervous system (Yamazaki, 1992). It works together with metabotropic glutamate receptors, ensuring the establishment of long-term potentiation (LTP), a process thought to be responsible for learning and memory (Zhuo et al., 2009). These functions are mediated by calcium entry through the NMDA receptor-associated channel which activates a number of Ca^{2+} -dependent enzymes that influence a wide variety of cellular components like cytoskeletal proteins or second messenger synthases like Ca^{2+} /calmodulin kinase II (CaMKII) (Strack et al., 1997; Bayer et al., 2001). However, over activation at NMDA receptors, triggers an excessive entry of Ca^{2+} , initiating a series of cytoplasmic and nuclear processes that promote neuronal cell death (Churn et al., 1995; Wroge et al., 2012).

6.1.1.2 NMDAR Encephalitis and other Disorders

The NMDAR have been associated with a variety of neurological disorders including ischemic brain damage and epilepsy, and, more speculatively with neurodegenerative disorders such as Alzheimers, Huntingtons chorea, and ALS (Waxman and Lynch, 2005; Ghasemi and Schachter, 2011).

Anti-NMDA receptor encephalitis has been recently identified as treatment responsive encephalitis associated with anti-NMDA receptor antibodies (Iizuka and Sakai, 2008). These antibodies bind to the NR1/NR2 heteromers of the NMDA receptors and antibody levels are known to correlate with the clinical severity of the disease (Irani and Vincent, 2011). Recent studies have demonstrated that these antibodies cause a reversible reduction in the numbers of cell-surface NMDA receptor clusters in post-synaptic dendrites, suggesting antibody mediated decreased NMDA receptor function (Iizuka, 2009).

NMDAR encephalitis is highly characteristic occurring in five stages: the prodromal phase with viral infection like symptoms, psychotic phase, unresponsive phase, hyperkinetic phase, and gradual recovery phase (Iizuka et al., 2010). Psychiatric manifestations are observed that upon progression lead to decrease of verbal output, catatonia, seizures, dyskinesias, hypoventilation and frequent autonomic instability (Ryan-N et al., 2011). In some cases it is a paraneoplastic syndrome usually affecting childbearing-age female with ovarian tumours, however, recent reports suggest a much higher incidence of nonparaneoplastic cases in males and children (Lebas et al., 2010; Hung et al., 2011). Treatment of anti-NMDAR encephalopathy includes immunotherapy and/or tumour removal. Anecdotal reports have revealed that recovery may be spontaneous without tumour resection but early tumour resection along with aggressive immunotherapy facilitates early functional recovery and 65% of patients with anti-NMDAR encephalopathy

exhibit full or near-full recovery (Suzuki et al., 2008). Most patients receive corticosteroids, IVIg or plasma exchange as first-line of immunotherapy (Dalmau et al., 2011). It is likely that patients who do not respond to one form of immunotherapy might respond to others regimens including plasmaphoresis, cyclophosphamide, and rituximab (Irani and Vincent, 2011).

Clinical features seen in EL coincide with the NMDAR receptor encephalitis which might make it a part of the spectrum of anti-NMDAR disorders. Dale et al. (2009) studied sera from paediatric patients with contemporary EL and found 10 sera out of 20 (from 2 males and 8 females, aged 1.3–13 years) and 6/6 cerebrospinal fluid samples positive for antibodies against the NMDA receptor. Different disease phenotypes were observed between the NMDA receptor positive and negative patients suggestive of different pathogenic targets. The antibody concentration in all cases was found to be higher in the serum than CSF (Dale et al., 2009).

Other studies have shown NMDAR hypofunction as a central component of the functional dysconnectivity as the most accepted model of symptoms seen in schizophrenia, a complex disorder with unknown aetiology. Zandi et al. (2010) tested the serum of 46 patients (63% schizophrenia, 15% psychosis not otherwise specified, 13% bipolar affective disorder, 4% schizoaffective disorder, 2% major depression with psychosis and 2% delusional disorder) for antibodies against NMDA receptor and found 4 schizophrenia patients positive for these antibodies (Zandi et al., 2010).

Studies have also implicated the NMDA receptor in the development of EAE the representative model of multiple sclerosis, with a particular role in the loss of blood brain barrier (BBB) function and recruitment of inflammatory cells (Paul and Bolton, 1995). A recent case study has also reported the presence of anti-NMDA antibody-positive limbic

encephalitis in a patient with multiple sclerosis. The simultaneous manifestation of both diseases has never been reported before and is suggestive of a possible link between these diseases or underlying, autoimmune responses that may be important for the development of autoimmune encephalitis (Uzawa et al., 2011).

6.1.2 Voltage Gated Potassium Channels (VGKC)

6.1.2.1 Background

Potassium channels are a ubiquitous and diverse family of membrane proteins present in both excitable cells such as neurons and nonexcitable cells (Heinemann and Hoshi, 2006). Members of this channel family play critical roles in cellular signalling processes regulating neurotransmitter release, heart rate, neuronal excitability, epithelial electrolyte transport, insulin secretion, smooth muscle contraction, and cell volume regulation (Shieh et al., 2000). Voltage-gated potassium channels (Kv channels) are transmembrane channels that allow rapid and selective flow of potassium ions across the cell membrane thereby generating and propagating electrical impulses in the nervous system (Sands et al., 2005).

Typically, vertebrate voltage-gated K⁺ channels are tetramers of two functionally and structurally independent domains: an ion conduction pore, and voltage-sensor domains. The ion conduction pore is composed of four subunits arranged symmetrically as a ring around the conduction pathway (Jiang et al., 2002; Khalili-Araghi et al., 2006). Voltage-sensor domains are positioned at the periphery of the channel and consist of four transmembrane segments (S1-S4) (Gandhi et al., 2002). The structural element responsible for the high selectivity of the channel is a sequence of five amino acids, TVGYG that is highly conserved among potassium channels, this forms the narrowest part of the channel, also known as the selectivity filter (Lu et al., 2001).

6.1.2.2 VGKC and Autoimmune disorders

Anti-voltage-gated potassium channel antibodies (anti-VGKC-Ab) are known to cause hyperexcitability of the CNS and peripheral nerve (Arimura et al., 2007). These antibodies are known to cause three rare neurological syndromes: neuromyotonia, limbic encephalitis and Morvan's syndrome although an increasing array of other associated neurological symptoms are now becoming recognised (Molloy et al., 2011).

Limbic encephalitis typically presents with subacute cognitive decline, seizures and behavioral disturbances like disorientation, anxiety, depression and hallucination (Derry et al., 2011). Paraneoplastic cases are rare unlike other anti-VGKC-Ab-associated neurological disorders. Current diagnostic criteria is based on neuropathological/neuroradiological evidence of a malignancy, usually small-cell lung cancer, testicular cancer, or thymoma (Graus et al., 2004). However, it is now recognised that in a small proportion of cases this syndrome is not paraneoplastic in nature (Buckley et al., 2001). Morvan syndrome is characterized by neurological symptoms involving the autonomic system (hyperhidrosis, urinary incontinence, and cardiac arrhythmia), peripheral nervous system (neuromyotonia), and the CNS (severe insomnia, hallucinations, impairment of short-term memory and epilepsy) (Vincent et al., 2004; Misawa and Mizusawa, 2010). Both VGKC-Ab-associated limbic encephalitis and Morvan syndrome are treatable conditions responding well to responding well to cortico-steroid therapy, plasma exchange and intravenous immunoglobulin (Vincent et al., 2004; Misawa and Mizusawa, 2010). Until recently, children with encephalitis were not routinely tested for VGKC antibodies and testing typically occurred only after an year or more of symptoms (Dale and Vincent, 2010).

Detection and measurement of VGKC Abs are useful in the diagnosis and management of autoimmune VGKC channelopathies and related neurological disorders. It has recently

become evident that the actual antigens involved in antibody binding are in fact proteins closely complexed with VGKC namely LGI-1 (leucine-rich glioma inactivated 1) and Caspr-2 (contactin-associated protein-like 2) and less frequently Contactin-2 (Irani and Vincent, 2011). Early clinical descriptions suggest that LGI-1 antibodies are mostly associated with the classic limbic encephalitis (Dalmau et al., 2010), whereas Caspr-2 antibodies are associated with both limbic encephalitis and neuromyotonia (Lancaster et al., 2011).

6.2 HYPOTHESIS AND AIMS:

The spectrum of neurologic manifestations and neoplasms associated with voltage-gated potassium channel autoimmunity is broader than previously recognized. Similarly pathogenic antibodies against the NMDAR have been implicated in a range of disorders; however, the full clinical spectrum and treatment responses are still unclear. Therefore, in the present study I analysed serum samples from a mixed cohort of patients with a range of neuropsychiatric movement disorders for the presence of anti-NMDAR and anti-VGKC antibodies.

6.3 MATERIALS AND METHODS

6.3.1 Patient Samples

A total of 46 patients samples were used for both NMDAR and VGKC assay. The cohort included sera from patients with TS (n=21), EL (n=11), chorea (n=6), dystonia (n=4) and PANDAS (n=4). The mean age of patients with TS was 11 years with an age range of 7-14 years. Only 2 out of the 21 TS patients were females. Patient material was obtained with informed consent and appropriate ethical review.

6.3.2 NMDAR Assay

The NMDAR assay is a cell based assay used for detection of anti-NMDA antibodies present in serum/CSF employing human embryonic kidney 293 cells (HEK293) transfected with NR1 and NR2B NMDAR subunits and co-transfected with EGFP (enhanced green fluorescent protein). The assay was carried out with Dr. Leslie Jacobson at the Weatherall Institute of Molecular Medicine, University of Oxford.

Day 1

3 coverslips of 13 mm diameter were put into each well of a 6 well plates using fine forceps. Approximately 2mL of poly-L-lysine (0.01% in PBS) was added to each well and incubated for 15-20 min. Following the incubation the supernatant was taken off and the plates were left to dry in the tissue culture hood with the lid open. 3×10^5 HEK293 cells in 2mL of Dulbecco's modified Eagle's medium (DMEM) with 10% foetal calf serum (FCS) and penicillin, streptomycin and amphotericin (DMEM+FCS+P/S) were seeded into each well and the plates were incubated for 24 hours at 37°C in a humidified chamber.

Day 2

Transfection of HEK cells with the NR1:NR2B glutamate receptor subunits was carried out with untagged-NR1 and NR2B cDNA at a ratio of 3:1. An EGFP expression vector was co-transfected to visualize cells taking-up cDNAs. DNA and polyethylenimine (PEI) mixture was prepared as described below:

4 µg DNA/well (NR1:NR2B in a ratio of 3:1)

1.5µL PE/well

50µL DMEM medium without supplements

The above mixture was incubated at RT for 10 min before adding it to the cells.

Day 3

500 μ M of ketamine was added to the cells 16 hs post- transfection. Ketamine is one of the most common glutamate receptor antagonists and therefore it blocks the NR1/NR2 subunits which become functional when transfected into cells. These functional subunits lead to cell apoptosis.

Day 4

Serum samples were diluted 1:20 in DMEM+HEPES+1% BSA (2.3 gms HEPES/500mL DMEM) to make up a total volume of 200 μ L/sample. The serum samples were then transferred to 24-well cell culture plates and a coverslip coated with NR1/NR2 transfected HEK cells was then placed in each well with a serum sample. The plates were then incubated for 60 minutes at RT in the dark. Following the incubation the supernatant was taken off and the wells were washed 2 x with DMEM with HEPES and 1 x with PBS. The cells were then fixed using 4 % paraformaldehyde (PFA) and incubated for 10 min at RT followed by 3X washes with DMEM+HEPES. 250 μ L of goat anti-human IgG, AlexaFluor 568 secondary antibody diluted 1:750 in DMEM+HEPES+BSA was then added and the samples were incubated for 45 min at RT in the dark. The samples were then washed 2X in DMEM+HEPES and 3X in PBS. Each coverslip (cell side down) was placed on a drop mounting medium containing DAPI (~ 20 μ L) on superfrost microscopic slides. The slides were then left to dry in the dark for atleast 3 h before viewing them under Leica DM 2000 fluorescent microscope. Pictures were taken using QCapturePro programme. The slides were then stored at 4°C.

6.3.3 VGKC Assay

The VGKC assay was carried out by Dr. Bethan Lang at the Weatherall Institute of Molecular Medicine, University of Oxford. In the VGKC autoantibody assay, antibodies in patient sera and controls are allowed to interact with detergent solubilised VGKCs extracted from rabbit brain tissue and complexed with ¹²⁵I-labelled α -dendrotoxin.

6.3.3.1 Preparation of whole rabbit brain homogenate

Whole rabbit brain was homogenised with 6X volumes of the solubilisation buffer containing protease inhibitors. An electric homogeniser was used for 3 x 10 seconds, returning the tissue/homogenate to ice in between pulses. The homogenate was then centrifuged at 17,000 x g at 4°C for 10 min following which the supernatant was discarded and the precipitate was re-suspended in an equal volume of SB (+ PIs) as the original tissue weight. The aliquots were stored at -80°C until required.

6.3.3.2 Preparing the VGKCs

2mLs dendrotoxin buffer pH7.12

500 μ L whole rabbit brain homogenate

0.05g Calbiochem high purity digitonin

5 μ L 0.1M PMSF

The buffer was warmed in a glass universal in a 37°C water bath and digitonin was added onto the surface of the buffer. Whole rabbit brain homogenate was then added together with PMSF and mixed carefully following incubation in the water bath at 37°C for a further 15 MIN . This mixture was then transferred to clear eppendorf/microcentrifuge tubes

without making bubbles and microfuged at 17,000 x g for 15 min at RT. The supernatant was then removed, which contained the soluble VGKC and the pellet discarded. The VGKCs in the supernatant were used directly to make the assay mix with the ^{125}I α -dendrotoxin (prepared as directed by the manufacturer). Generally, 2mLs VGKC from supernatant + 2mLs PTX buffer + ^{125}I α -Dendrotoxin to give ~ 45 – 50,000 cpm (measured counts per minute) for 50 μL of the final mixture is used. This was done only when the VGKC supernatant was ready and was followed by direct incubation at RT for 15 min, and then transferred to 4°C. The samples were vortexed and then microfuged at 17,000 x g for 5 min just before aliquoting the appropriate amount (50 μL) into the final assay incubation tube. At least 3 healthy control sera and a variety of known positive VGKC sera were used in each VGKC assay.

6.3.3.3 Setting up the assay

The diluted and microfuged control and test samples were aliquoted into the final assay tubes following the addition of the assay mix. For screening the serum samples a 1:1 dilution was used: 50 μL diluted serum + 50 μL assay mix. The mixture was incubated overnight at 4°C.

6.3.3.4 Precipitation with anti-human IgG

50 μL of sheep anti-human IgG (Fc) was used with 5 μL serum and incubated at room temperature for approximately 80 min. Once precipitated, 500 μL PTX was added to each tube immediately before microfugation at 17,000 x g for 5 min. The supernatants were then aspirated immediately and the pellets washed twice with 500 μL PTX.

6.3.3.5 Counting the radioactivity in the pellet

For screening an assay of 5 μ L the mean cpm of the healthy controls was subtracted from the cpm of all other samples, divided by 10 and this equalled concentration of anti-VGKC antibodies present in the sample.

6.4 RESULTS

6.4.1 Anti-NMDAR Antibodies

The NMDAR assay was performed to analyse serum samples for presence of antibodies against the NMDA receptor using HEK293 cells transfected with NR1:NR2B subunits and co-transfected with EGFP. According to the number of cells stained and intensity of staining the samples were scored between 0-4 (0= negative, 1= weakly positive, 4= strongly positive). The samples were used at a 1:20 dilution with repeats at various titrations like 1:100 and 1:500. The sera were analysed against the glycine receptor as a positive control. 4 out of the 46 serum samples used in this study tested positive for antibodies against the NMDAR receptor. All 4 serum samples came from male patients diagnosed with TS with ages ranging from 8 to 14 years.

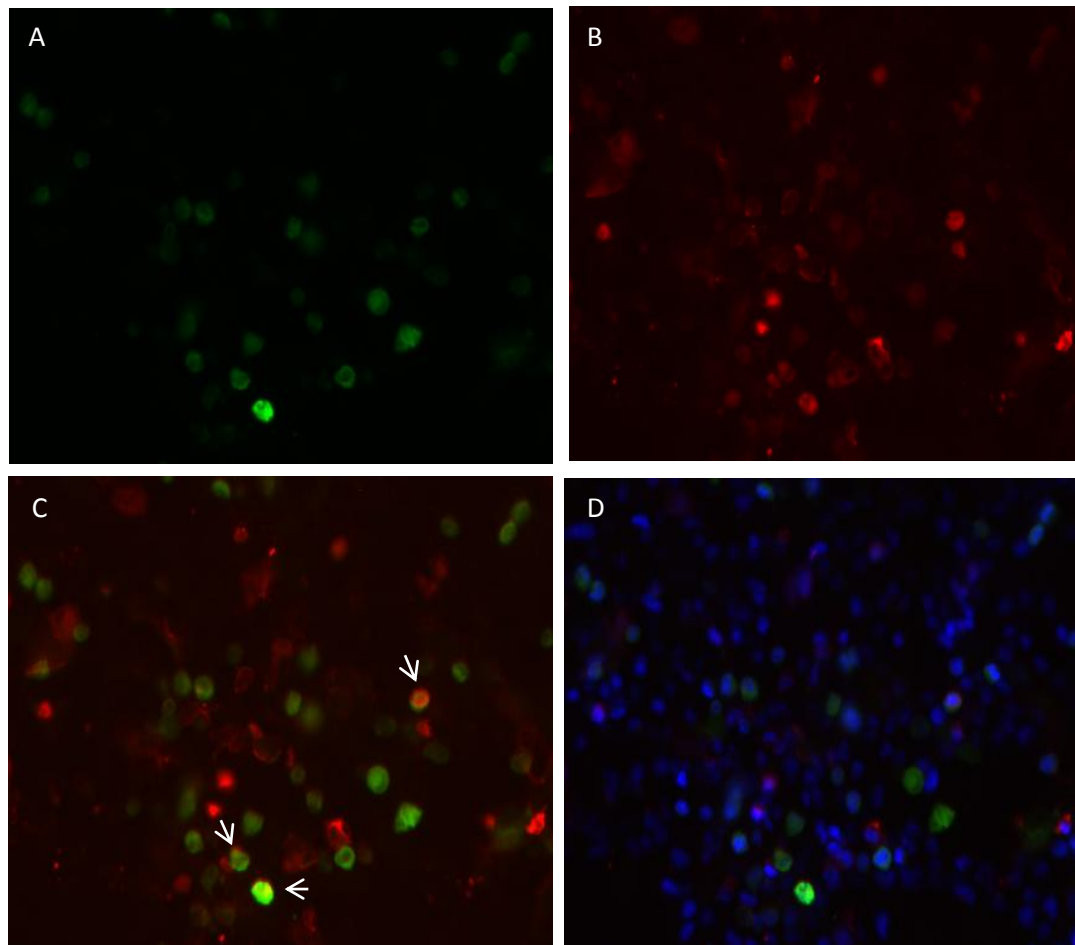


Figure 6.1: Fluorescent 40x images of serum found positive for anti-NMDAR antibodies.

The NMDAR assay was performed using HEK293 cells transfected with NR1:NR2B NMDAR subunits and co-transfected with EGFP. The images above are from a serum sample found positive for antibodies against the NMDAR. The serum came from a 14 year old male diagnosed with TS. **(A)** EGFP staining. **(B)** Positive staining for NR1:NR2B NMDAR subunits. **(C)** Merged image of A&B showing co-localization of EGFP and NMDAR subunits. **(D)** Merged image with DAPI stained nuclei showing co-localization of NR1:NR2B subunits, EGFP and DAPI.

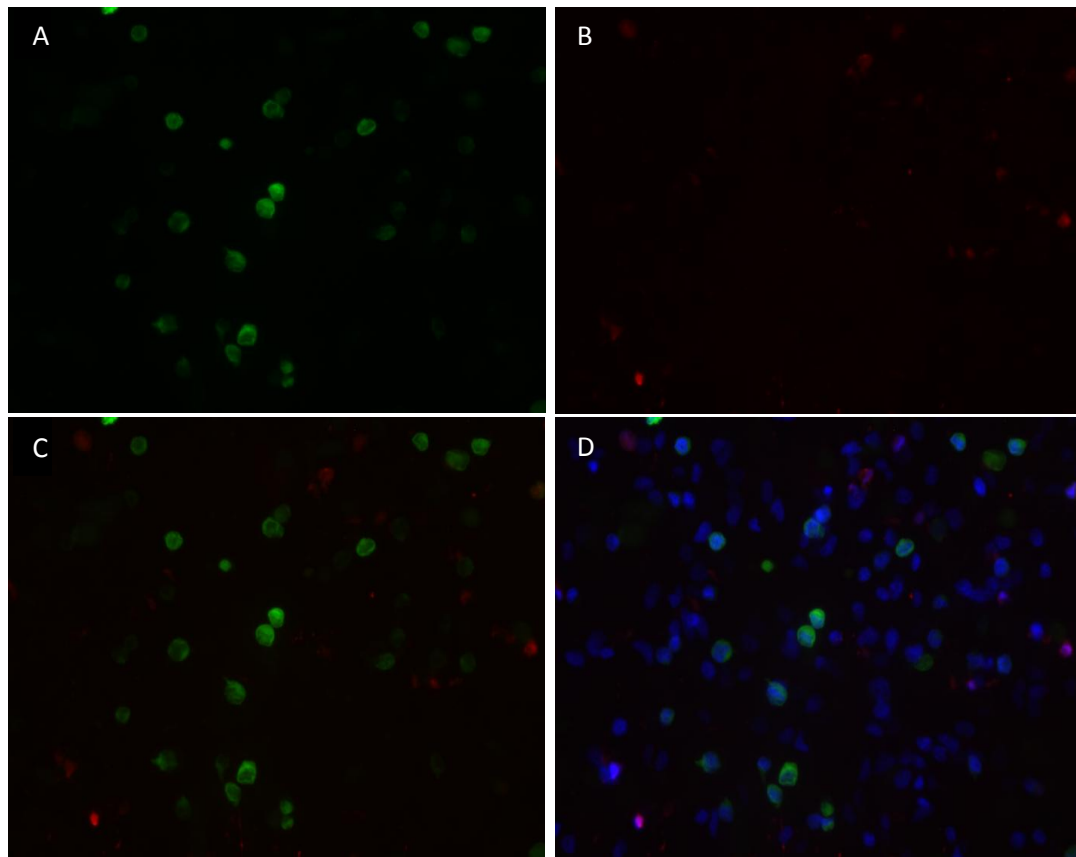


Figure 6.2: *Fluorescent 40x images of serum found negative for anti-NMDAR antibodies.*

The NMDAR assay was performed using HEK293 cells transfected with NR1:NR2B NMDAR subunits and co-transfected with EGFP. The images above are from a serum sample found negative for antibodies against the NMDAR. (A) EGFP staining. (B) Negative staining for NR1:NR2B NMDAR subunits. (C) Merged image of A&B showing co-localization of EGFP and NMDAR subunits. (D) Merged image with DAPI stained nuclei showing co-localization of NR1:NR2B subunits, EGFP and DAPI.

6.4.2 Anti-VGKC Antibodies

All 46 serum samples were also tested for antibodies against the voltage gated potassium channel. In the VGKC autoantibody assay, antibodies in patient sera and controls are allowed to interact with detergent solubilised VGKCs extracted from rabbit brain tissue and complexed with ¹²⁵I-labelled α -dendrotoxin. High titres of anti-VGKC were seen in serum from 2 patients with measured counts per minute of 2566 and 215. Both these patients were diagnosed with chorea.

6.5 DISCUSSION

In the last decade significant advances have been made in understanding the spectrum of probable antibody-mediated central nervous system diseases. With the development of assays for detection of antibodies against cell surface neuronal proteins like the NMDA receptor and VGKC the diagnosis of these disorders has now become feasible. The full clinical spectrum of antibody mediated CNS disorders is now only likely to widen with their increasing recognition.

Antibodies against the NMDA receptor have now been implicated in a range of disorders like classical limbic encephalitis, psychosis and epilepsy and more recently also found in patients with multiple sclerosis, EL and schizophrenia. In the present study the presence of anti-NMDAR antibodies in the sera of 4 TS patients has been demonstrated. This finding to the best of our knowledge is the first to show the presence of anti-NMDAR antibodies in a minority of subjects with TS. TS is comorbid with multiple conditions like OCD, ADHD, depression and anxiety disorders (Martino et al., 2009) so an understanding into its pathogenic mechanism would have major implications in the field of neuropsychiatry. Previously TS and OCD were considered to be disorders mediated by serotonin and dopamine mechanisms but now the role of glutamate is becoming important given its

pivotal role in neurotransmission and neuronal development (Pittenger et al., 2006). A study by McGrath et al. (2000) has demonstrated using D1CT model of comorbid TS+OCD, that glutamatergic drugs such as MK-801, a non-competitive NMDA receptor antagonist, indirectly stimulate the cortical-limbic glutamate output and aggravate abnormal behaviour (repetitive climbing and leaping) (McGrath et al., 2000). Further evidence implicating glutamatergic transmission in OCD has come from rat model of OCD in which Albelda et al. (2010) have shown that D-cycloserine (15mg/kg), a partial NMDA agonist can selectively decrease compulsive lever pressing suggesting that activation of NMDA receptors may have an anti-compulsive effect in OCD patients (Albelda et al., 2010).

VGKC given their pivotal role in various physiological processes including neuronal signalling are increasingly being elucidated as molecular targets in a number of pathophysiologic states, and they continue to trigger considerable enthusiasm as drug targets. Anti-VGKC antibodies have been suggested to cross the BBB and act centrally, binding predominantly to thalamic and striatal neurons causing encephalopathic and autonomic features (Irani and Vincent, 2011). In the present study anti-VGKC antibodies were detected in 2 serum samples, both of which came from patients with chorea. Interestingly, testing for anti-VGKC antibodies have become part of the investigation of patients with unexplained subacute onset of epilepsy, memory or cognitive problems, or peripheral nerve hyperexcitability syndrome.

Increasing amount of evidence both *in vitro* and *in vivo* is pointing towards the pathogenicity of antibodies against cell surface proteins. The production of these antibodies is probably the result of a combination of factors such as genetic predisposition, presence of a systemic tumor or molecular mimicry caused by a prodromal infection. Irrespective of the cause, until now in most cases, the associated syndromes have

been responsive to immunotherapy, which highlights the importance of their detection in patients with otherwise unexplained encephalopathic symptoms. Autoimmune diseases have previously been studied on the basis of the affected organ, looking for a single causative factor, however, in the recent years the focus has changed to a more cross-disciplinary approach of these multifactorial diseases, with a view to providing a better understanding of the common mechanisms underlying their pathogenesis. The aim now is to tailor diagnosis and treatment by establishing these syndromes in terms of their full range of clinical presentations, frequency, means of early recognition, and optimal immunotherapy.

CHAPTER 7

CONCLUSIONS AND FUTURE WORK

7.1 OVERVIEW

In the last decade significant advances have been made in understanding the spectrum of probable antibody-mediated central nervous system diseases. A complex interplay between an array of both environmental and genetic factors underlie these disorders. The present study is focussed on unravelling the complex pathogenesis of neurological and neuropsychiatric disorders that have been linked to GABHS like SC, TS, and EL.

Although the pathophysiology of post-streptococcal disorders is not completely understood, antigenic mimicry is proposed to be the triggering cause of autoimmunity (Hahn et al., 2005, David Greenwood, 2007). It is a possibility that GABHS needs to be the initial autoimmunity-inciting event but that subsequent symptom exacerbations might be triggered by viruses, other bacteria, or non-infectious immunologic responses. In the pathogenesis of these diseases, host factors may also be of critical importance. Gender appears to be a risk factor, as three-quarters of the PANDAS are males whereas SC is more prevalent amongst females. The age of the host also may determine susceptibility as it is known that rheumatic fever and SC are quite rare after puberty. It is also possible that the post-pubertal decrease in incidence is related to the fact that the rate of GABHS infections falls dramatically around the age of 12, presumably because the child has developed antibodies against the conserved portion of the M-protein. Genetic control of the immune response may contribute to differential vulnerability to PANDAS. Mouse genetic models suggest that the response to infectious pathogens is strain specific. Different strains have different qualitatively and quantitatively response that leads to very different clinical outcomes.

To conclude the entity of CNS diseases falling under the post-streptococcal umbrella is still controversial. A multidimensional approach was undertaken to understand the complex dynamics of these disorders as briefly summarised below.

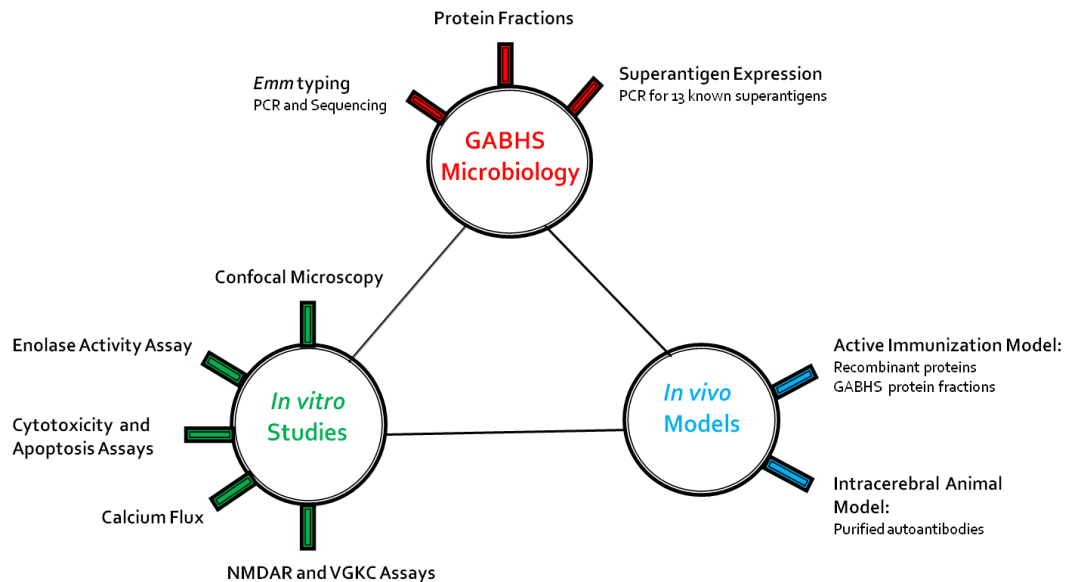


Figure 7.1: An overview of the work carried out during the PhD

This PhD provides a brief insight into this spectrum of disorders. The synopsis of the results obtained is briefly described below together with the potential future work I would like to perform working as a post-doctoral scientist.

7.2 GABHS

7.2.1 Patient Vs Control Phenotype

Among the various factors involved in the virulence of GABHS, the M protein and streptococcal superantigens have been the focus of interest because of their potential role in both autoimmunity and manifestations in acute infections. In the present study GABHS samples from both patients and controls were typed for the *emm* gene and the presence of SAg. One aim was to determine whether *emm* types and SAg were more prevalent in patients diagnosed with suspected post-streptococcal neuropsychiatric disorders as

compared to neurological controls and if there was any co-relation between the two virulence factors. Our results showed that certain *emm* types were found only in the patient population like *emm4*, *emm11* and *emm3*. Different *emm* types tend to predominate in different age groups and geographical locations. The combination of factors results in a complex epidemiologic situation for GABHS infections requiring a deeper understanding of geographic and temporal variation in *emm* types. Once again a range of superantigens were observed to be present only in the patient isolates like *speA*, *H*, *I*, *J*, *K*, *L* and *M* which might be enhancing the pathogenicity of these isolates. Whilst studying the link between the two factors a few observations were made like *speL* and *M* were found only in *emm* type 4 patient samples and not controls. Certain combinations of superantigen genes are found to be more common and the majority of *emm* types showed restricted superantigen profiles.

The pattern seen in our study has been consistent with previous findings in the field making it possible for us to draw certain definitive conclusions about the co-relation between the two virulence factors. However, the importance and relevance of these relationships needs further investigation by clarifying their role in the outcome of infection with GABHS. The data presented will be taken forward by analysing a large cohort of both patient and control samples as it could be of value for preventive work, including ongoing attempts at creating vaccine prophylaxis against GABHS disease.

7.2.2 Is GABHS linked to this group of disorders?

Only a limited number of animal models have been set up using GABHS to confirm its link with the onset of a spectrum of neuropsychiatric disorders in children. Animal models to test the hypothesis that an immune response to GABHS can result in behavioural abnormalities was carried out by Hoffman et al. (2004), Yaddanapudi et al. (2009) and

Brimber et al. (2012). They observed deficits in motor coordination, learning/memory and social interaction in PANDAS mice, replicating more complex aspects of human disease. In our active immunization mouse model the animals exhibited decreased activity in the open field activity chamber. Although no motor deficits were observed using the rotor rod testing. The lack of detailed behavioural testing in our study may have prevented us from picking up subtle behavioural and locomotor abnormalities reminiscent of these disorders.

7.2.2.1 Future Work

This animal model would be taken further by injecting mice with purified M proteins (perhaps specific serotypes) or/and superantigens which would help us establish a link between streptococcus and these disorders together with pin pointing the proteins causing the virulence. Also a number of behavioural tests will be set up to better evaluate these animals. In addition videotaping the animals would help observe subtle signs and changes in behaviour.

In an *in vitro* setting I would like to create a GABHS infection model using cultured phagocytes to study dose-dependent apoptosis. GABHS have been seen to induce apoptosis in human neutrophils (Kobayashi et al., 2003) although the specific virulence factor(s) involved and effects on caspase activation have not yet been explored. I could therefore use purified SAg and M proteins to study their individual pathogenic effect on cellular and sub-cellular targets.

7.3 AUTOANTIBODIES

7.3.1 *In vivo* effect

Previous studies have shown the effects of infusing serum immunoglobulin from patients with PANDAS into rat striatum found an increase in stereotypical movements compared to control antibodies (Hallett et al., 2000; Taylor et al., 2002). However, another group using

the same methods failed to reproduce the results (Loiselle et al., 2004). The present study is the first of its kind to create an intra-cerebral animal model of neuropsychiatric disorders like EL. In the present study it was aimed to determine the specificity of anti-basal ganglia antibodies and hence compare the effects of antibodies from both EL and dystonia patients. A varied phenotype was observed in the two cohorts with some common features like rigidity. The development of symptoms in mice after induction of autoantibodies representative of the disease in question confirms the pathogenicity and specificity of these antibodies. Although part of the same disease spectrum antibody we are possibly dealing with variable cellular and sub-cellular targets in this group of disorders. Given its novelty the present animal model only forms the basis for a much larger experiment.

7.3.1.1 Future work

Future work will involve injecting animals specifically into the basal ganglia which will be achieved by using a stereotactic frame. Additionally, antibodies from patients with TS would be used to determine the development of any stereotypies and episodic utterances that have been proposed as a model of motor and vocal tics. Also affinity purified antibodies against the known autoantigens especially enolase from sera of patients would be used to compare its effect with those seen with the commercial monoclonal antibody. electrophysiological experiments would be performed to rule out the occurrence of epileptic seizures, and confirm that the stereotypical movements seen in the mice are indeed representative of the disorders in question.

7.3.2 *In vitro* effect

It was hypothesized that an antibody-mediated autoimmune mechanism as well as bacterial infection contributed to the pathogenesis of neuropsychiatric movement

disorders like TS and SC. Hence the effect of patient and control antibodies was studied on both neuronal and non-neuronal cells in terms of cytotoxicity, apoptosis and intracellular calcium flux. A significant increase in both cytotoxicity and apoptosis was observed in response to patient antibodies highlighting their pathogenic effect. The cytotoxic and apoptotic effect of patient antibodies was consistent in both undifferentiated and PC12 differentiated into neurons. The main form of enolase dimer on undifferentiated cells is the $\alpha\alpha$ -homodimer (non-neuronal enolase) (Vinores et al., 1981). A change from non-neuronal to neuron specific enolase ($\alpha\gamma$ and $\gamma\gamma$ –dimer) during PC12 differentiation into neurons has also been documented (Schemedel et al., 1980). Additionally patient antibodies caused a significant increase in intracellular calcium flux in neuronal cells which could be responsible for activation of a number of enzymes, including phospholipases, endonucleases, and proteases which lead to damage of structures like the components of DNA and the cytoskeleton (McConkey and Nutt, 2001; Gronski et al., 2009) thereby leading to neuronal dysfunction.

7.3.2.1 Future work

In the present study it was not possible to perform an ATP assay due to technical difficulties. As part of the future studies I would like to determine the ATP/ADP ratios using a luciferase chemiluminescence assay (Pocock & Nicholls. 1998) to determine whether energy metabolism is also altered. ATP levels need to be assessed since apoptosis may be triggered upstream by perturbations in ATP, in particular a fall in ATP. It is also possible that an increase in ATP in some instances given current suggestions that cell surface glycolytic enzymes may actually serve to generate energy at the membrane (Dubinsky et al., 1998).

Although an effect of autoantibodies from patients on calcium flux was demonstrated, an identification of target receptor/channel is still pending that would help understand the downstream alterations in signal transduction. Some initial studies with patient IgG have

indicated decreased calcium responses in cultured neurones to a depolarising stimulus. In cultured neurones, enhanced intracellular free calcium provides trophic functions for neuritogenesis and gene transcription, particularly via the NMDA receptor and L-type calcium channel (Helton et al., 2005). In my future experiments I would determine the effects of purified monospecific antibodies on KCl-evoked calcium responses as well as NMDA stimulated calcium responses. Single cell Ca^{2+} responses can then be measured by fluorescence imaging as previously described (Evans & Pocock, 1999; Pocock and Evans 2000).

7.3.3 Other Potential Targets

Antibodies against the NMDA receptor have now been implicated in a range of disorders like classical limbic encephalitis, psychosis and epilepsy and more recently also found in patients with multiple sclerosis, EL and schizophrenia. In the present study I have demonstrated the presence of anti-NMDAR antibodies in the serum of 4 Tourette's patients. Previously TS and OCD were considered to be disorders mediated by serotonin and dopamine mechanisms but now the role of glutamate is becoming plausible given its pivotal role in neurotransmission and neuronal development (Pittenger et al., 2006). Also since testing for anti-VGKC antibodies have become part of the investigation of patients with unexplained subacute onset of epilepsy, memory or cognitive problems, or peripheral nerve hyperexcitability syndrome I tested our samples for anti-VGKC antibodies which were detected in 2 serum samples, both of which came from patients diagnosed with chorea.

7.3.3.1 Future work

Future studies involve testing this cohort of samples for presence of anti-dopamine receptor antibodies since in a rat model of PANDAS described recently alterations in dopamine and glutamate levels were seen in the cortex and basal ganglia. Furthermore, the

motor symptoms were found to improve by the D2 blocker haloperidol and the selective serotonin reuptake inhibitor paroxetine, respectively, these are the drugs used to treat motor symptoms and compulsions in streptococcal-related neuropsychiatric disorders (Brimberg et al., 2012).

7.4 ENOLASE

Enolase isoforms α and γ and their dimmers are some of the known targets of anti-neuronal antibodies (Dale et al., 2004; 2006). In the present study the role of enolase as a target autoantigen in postulated post-streptococcal CNS disorders was confirmed. A two way approach was adopted to confirm this, firstly through an animal model where I direct infusion of mAb against enolase into mice and secondly in an *in vitro* setting by assessing the action of antibodies from patients on the functioning of this enzyme.

7.4.1 *In vivo* proof

I worked on the hypothesis that if enolase indeed is a one of the autoantigen in this group of disorders then injecting mice with monoclonal antibody (mAb) against the protein should also result in stereotypical movements similar to those seen in human subjects. Intra-cerebral induction of mAb against NSE did result in a phenotype very similar to that seen in mice injected with patient antibodies exhibiting classical symptoms of tremor and rigidity. The pathogenic effect was consistent even at ten-fold lower antibody concentration. The results of this experiment strongly implicate NSE as being one of the antibody targets in this spectrum of neuropsychiatric movement disorders.

7.4.1.1 Future work

Future work will involve affinity purification of antibodies from patient sera using the recombinant protein enolase. These antibodies would then be stereotactically injected into the mouse basal ganglia.

7.4.2 *In vitro* proof

Fluorocytometric analysis has also revealed that anti-streptococcal enolase antibodies react with the enolase expressed on the surface of hematopoietic cells (Fontan et al., 2000). Keeping our results from the animal model and previous findings in mind I developed an enolase enzymatic assay to test sera for antibodies against enolase. There is only a poor influx of the enolase educt 2-phosphoglycerate (DPG) into the cells (Hamasaki et al., 1978) so that the main enolase activity measured in this assay comes from the surface enolase and released enolase. A marked inhibition of the enzymatic activity of enolase was observed after the addition of sera antibodies from patients as opposed to healthy controls.

7.4.2.1 Future work

Future work revolves around further refining the enolase enzymatic assay where I need to better define threshold values for both patient and control populations such that it can be introduced as a diagnostic screening test to detect the presence of anti-enolase antibodies.

7.4.3 Membrane expression of enolase

After confirmation that autoantibodies target cell surface enolase my next working hypothesis was where could this enzyme be expressed? It was postulated that since Na^+/K^+ -ATPase interacts with soluble, membrane and structural proteins that regulate the ion-pumping function and signal-transducing functions to the Na^+/K^+ -ATPase (Kaplan JH, 2005;

Li and Xie, 2009) enolase might be one of the associated proteins. Following this hypothesis undifferentiated and PC12 cells differentiated into neurons were stained for enolase and Na⁺/K⁺ -ATPase and found the two to be co-expressed.

7.5.3.1 Future Work

Diffused staining was repeatedly observed despite using various methods of cell fixation and blocking. Hence these experiment need to be repeated with a new set of antibodies and other neuronal cell lines. As mentioned earlier I also postulated that both enolase and Na⁺/K⁺-ATPase might be present on lipid rafts which are glycolipoprotein microdomains present in the plasma membrane. Future experiments would involve staining the rafts with fluorophores conjugated to cholera-toxin B-subunit, which binds to the raft constituent

7.5 CONCLUSIONS

In summary TS, SC, EL and other ABGA-associated disorders are still an emerging clinical entity, with major implications for neuropsychiatry. Establishing this group of disorders as “true” autoimmune diseases marks the first step towards future therapeutic strategies. All experiments conducted during the course of this PhD form the initial steps to understand the complex aetiology of these disorders. Further work needs to be done to disentangle the interplay between environmental factors and genetic background and translate these findings into clinical applications by developing disease prediction models and investigation of a treatment strategy. All the future work will be covered as part of a grant received by **European Multicentre Tics in Children Studies (EMTICS)** which is project funded by the EU programme FP7 combining 27 partners from 11 different countries. This programme aims to identify the genetic and environmental susceptibility factors of tic disorders leading to a better understanding of the underlying mechanisms, especially by elucidating the role of autoimmunity and infections.

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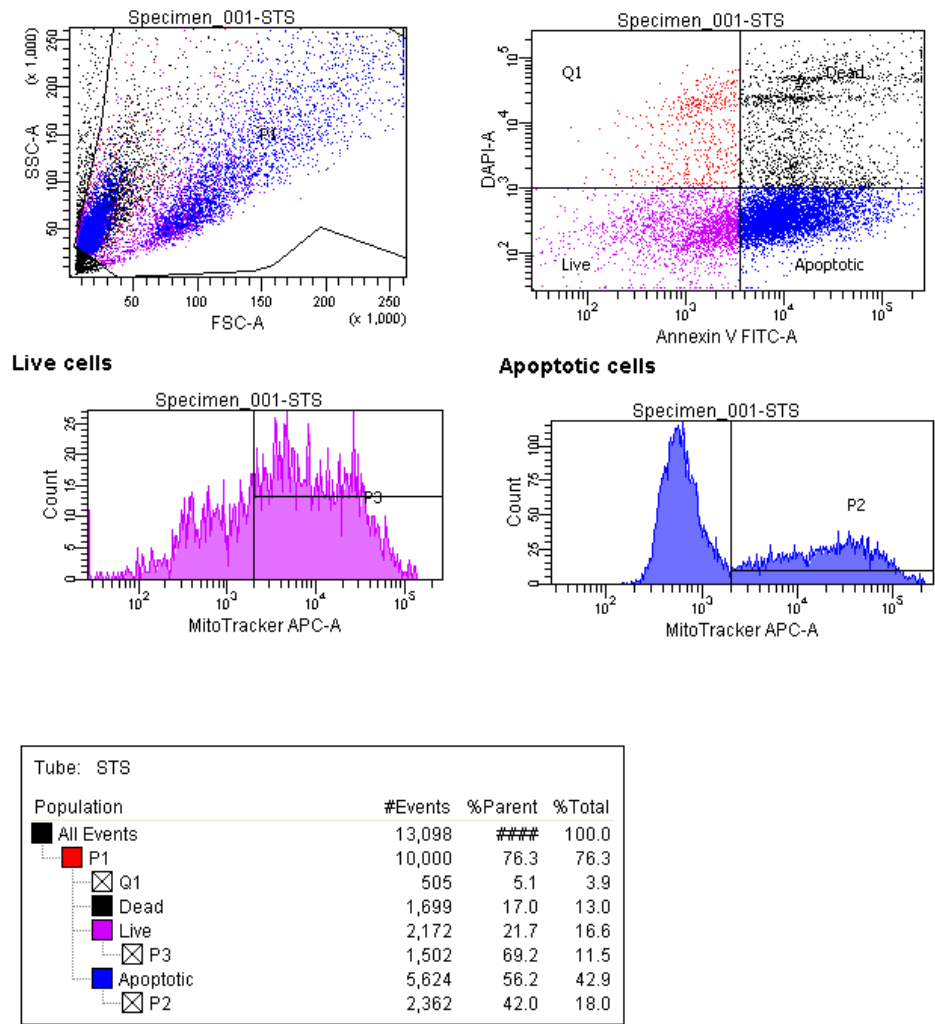
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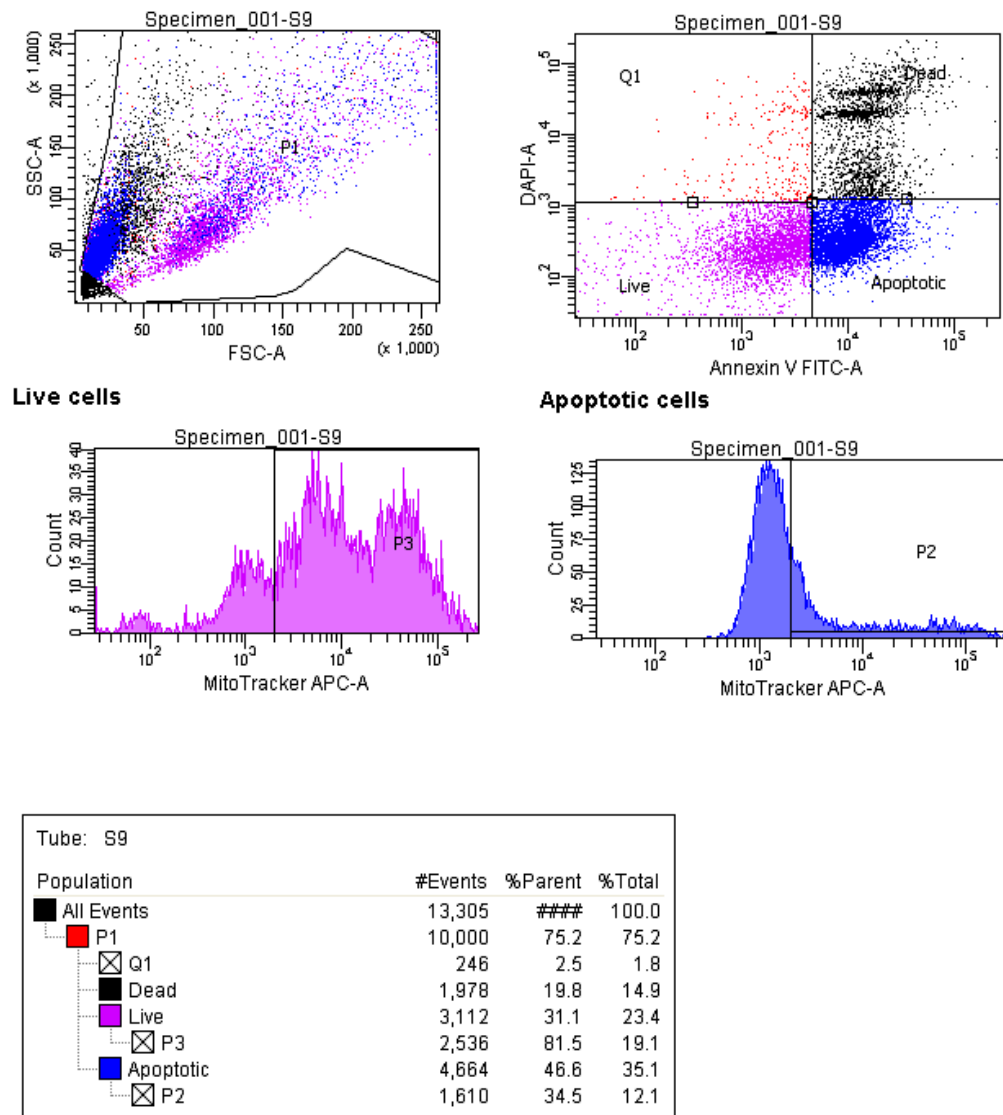
APPENDIX
SUPPLEMENTARY APOPTOSIS DATA

FACS: ANNEXIN V APOPTOSIS

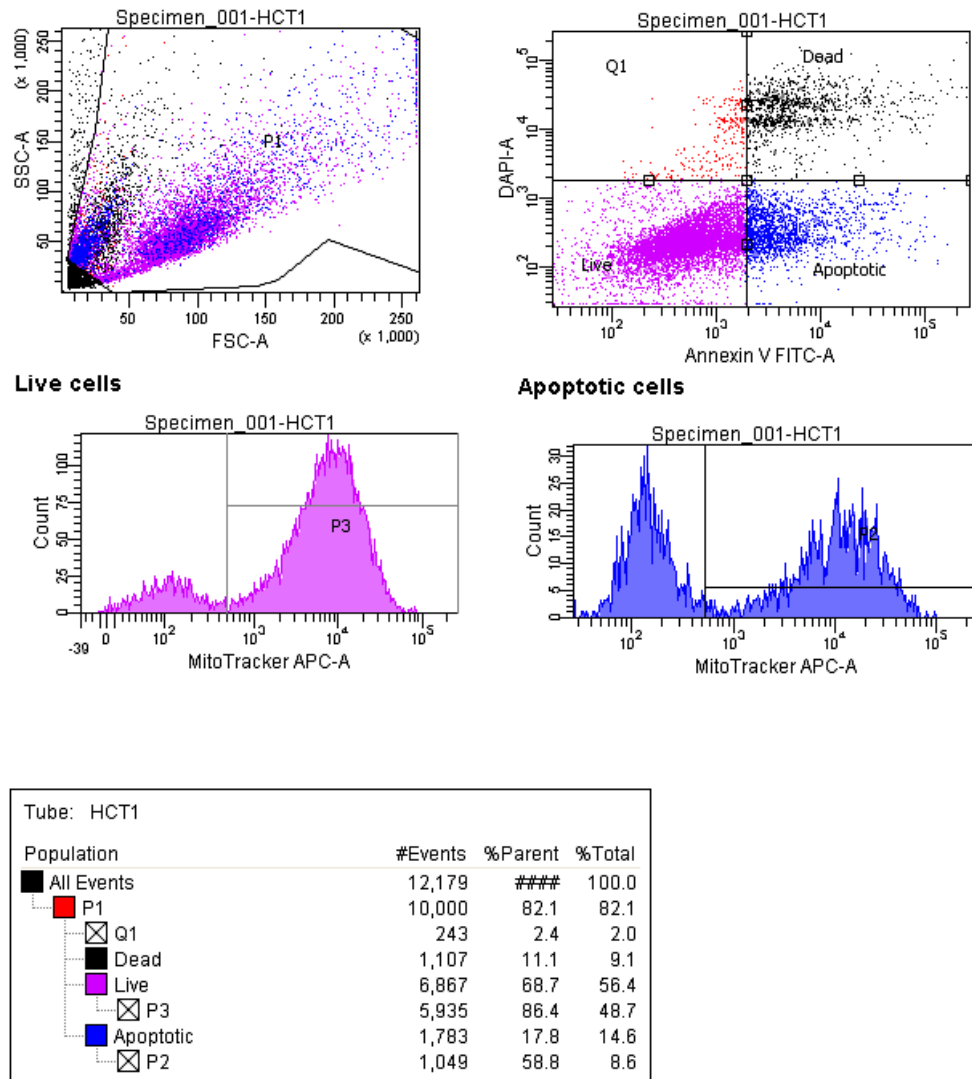
Undifferentiated PC12 cells: Human Samples



Supplementary Figure 1: FACS image for live and apoptotic undifferentiated PC12 cells after treatment with staurosporin (positive control). The above flow cytometry images show the percentage of live and apoptotic undifferentiated PC12 cells after treatment with staurosporin (1 $\mu\text{g/mL}$) a known inducer of apoptosis. 56.2% of cells were found to undergo apoptosis after addition of STS. Only 21.7% cells were found to be alive out of which 69.2% had intact mitochondrial function.

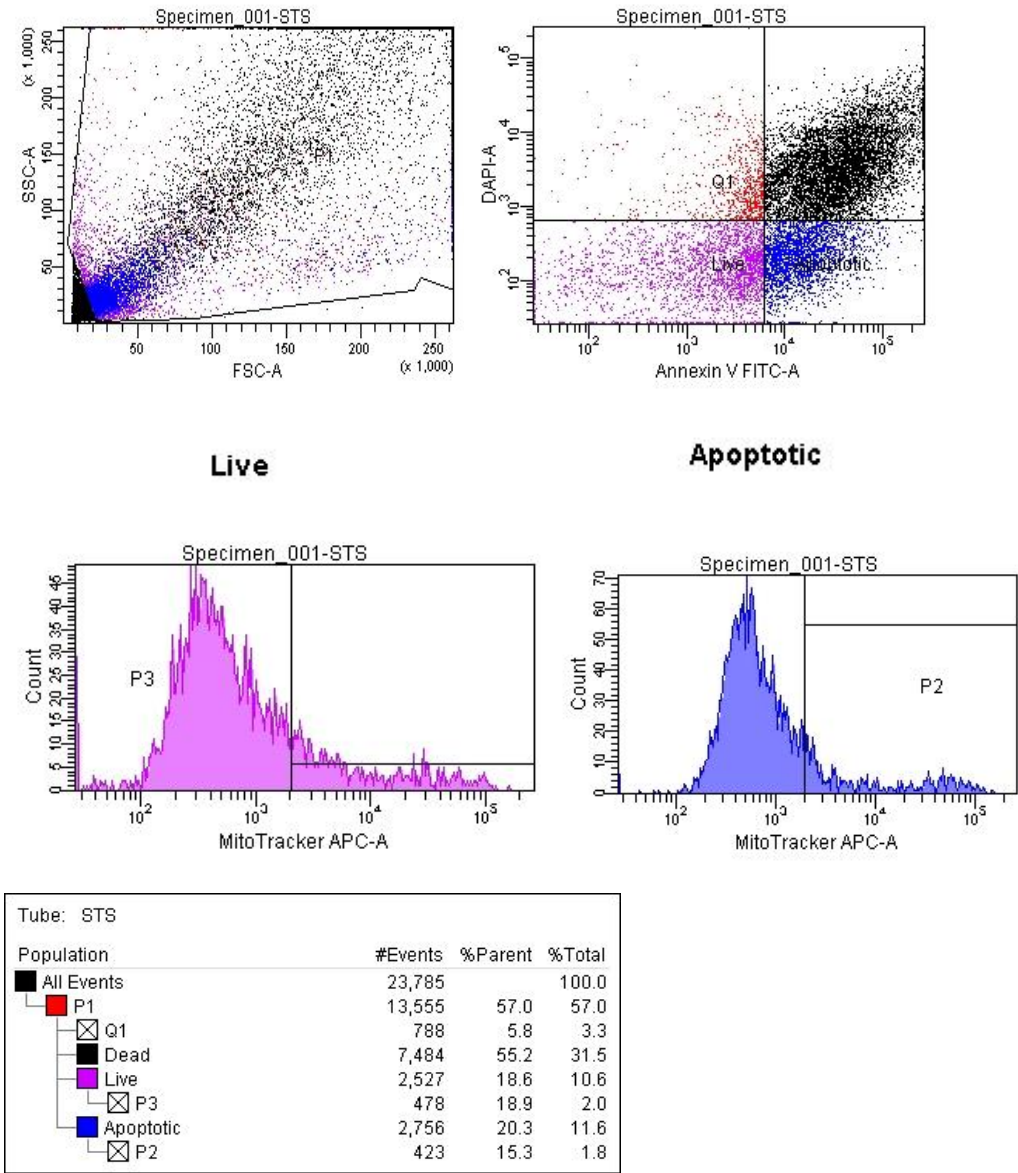


Supplementary Figure 2: FACS image for live and apoptotic undifferentiated PC12 cells after treatment with sera from a TS patient. The above flow cytometry images show the percentage of live and apoptotic undifferentiated PC12 cells after treatment with 1:100 sera from a TS patient. 46.6% of cells were found to undergo apoptosis after addition of sera antibodies. Only 31.1% cells were found to be alive out of which 81.5% had intact mitochondrial function.

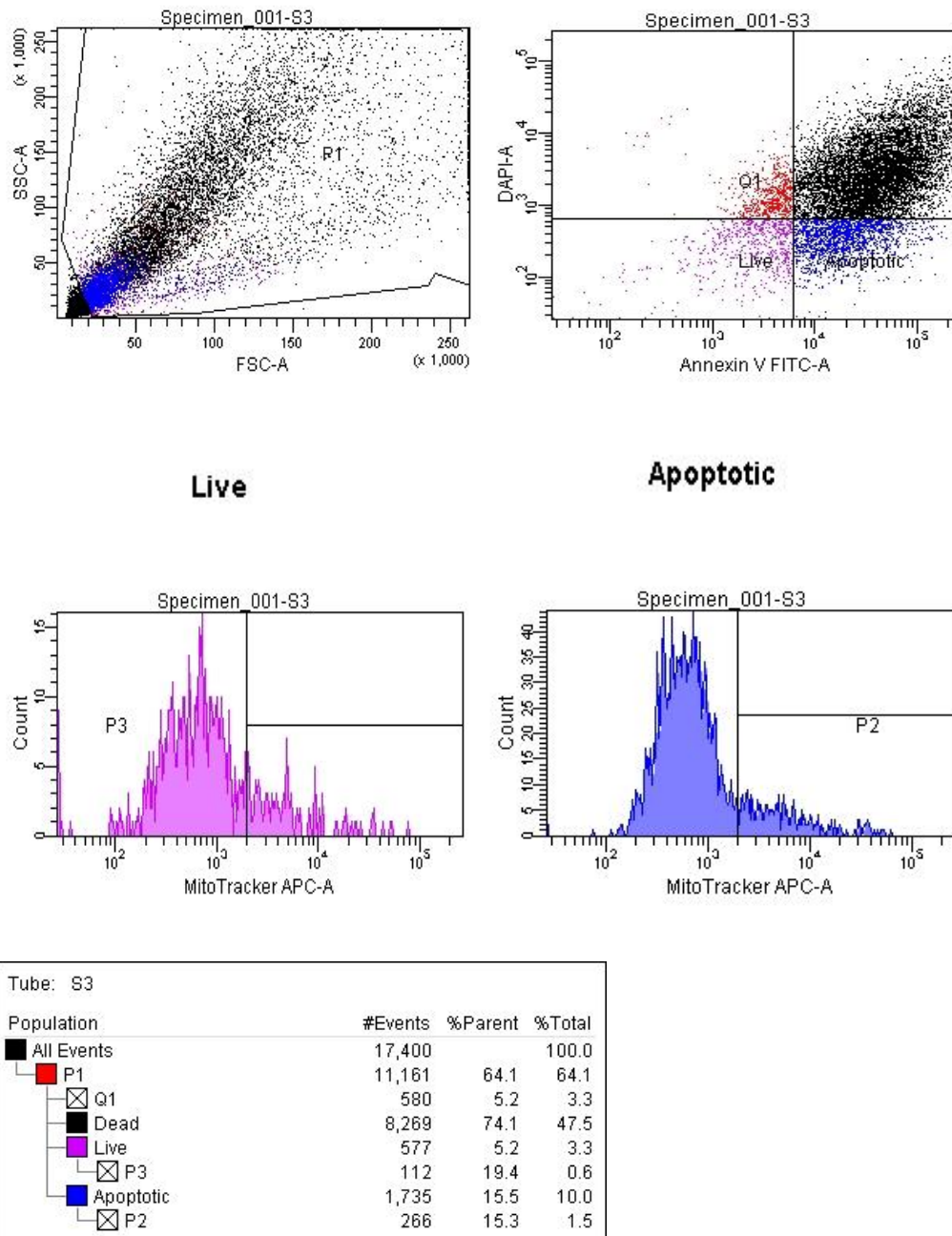


Supplementary Figure 3: FACS image for live and apoptotic undifferentiated PC12 cells after treatment with sera from a healthy control. The above flow cytometric images show the percentage of live and apoptotic undifferentiated PC12 cells after treatment with 1:100 sera from a healthy control. Only 17.6% of cells were found to undergo apoptosis after addition of control sera antibodies. 68.7% cells were found to be alive out of which 86.4% had intact mitochondrial function.

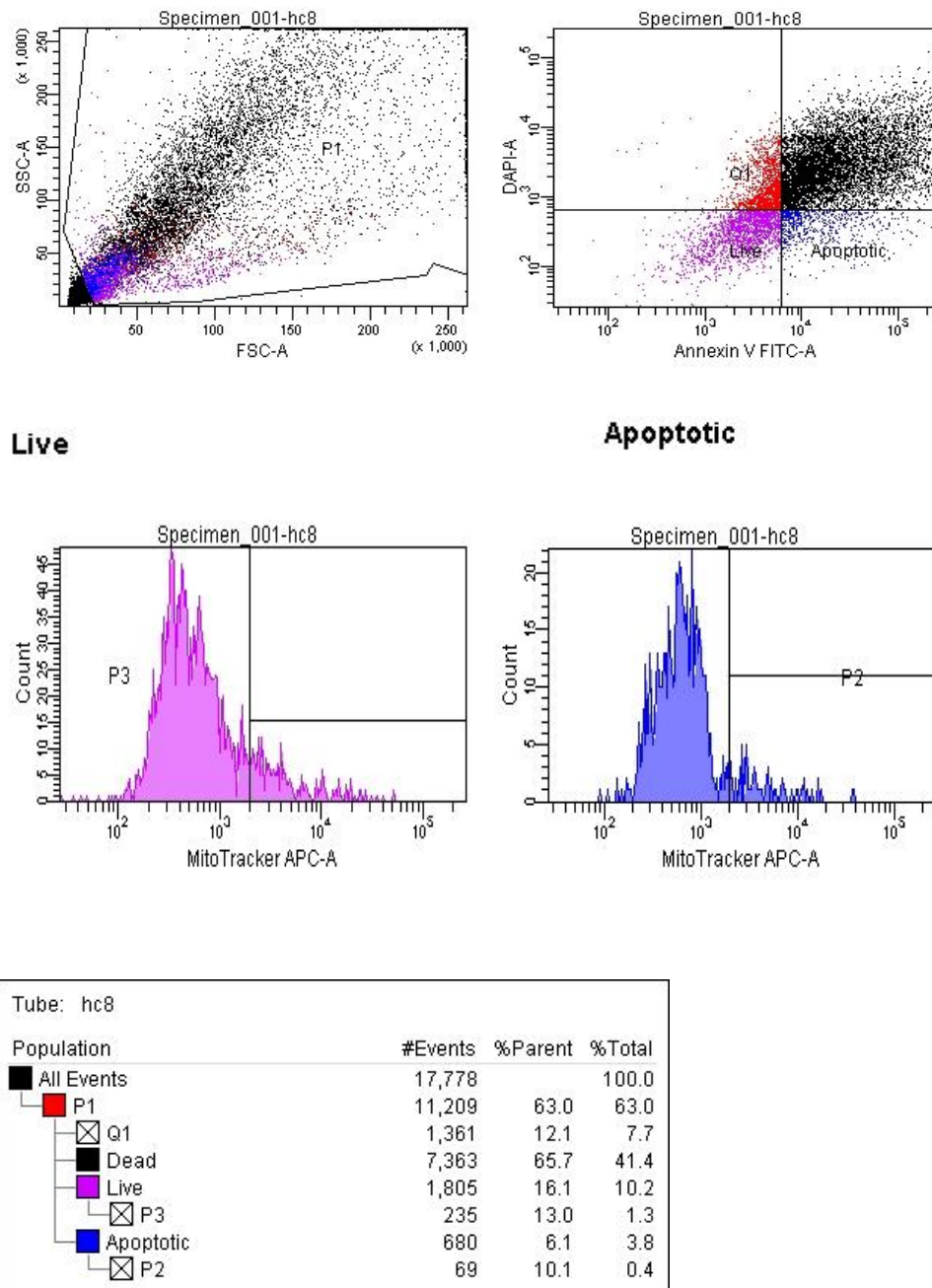
Differentiated PC12: Human Samples



Supplementary Figure 4: FACS image for live and apoptotic differentiated PC12 cells after treatment with staurosporin (positive control). Supplementary figure 4: The above flow cytometry images show the percentage of live and apoptotic PC12 cells differentiated into neurons after treatment with staurosporin (1 $\mu\text{g/mL}$) a known inducer of apoptosis. 20.3% of cells were found to undergo apoptosis after addition of STS. 18.6% cells were found to be alive out of which only 18.9% had intact mitochondrial function.

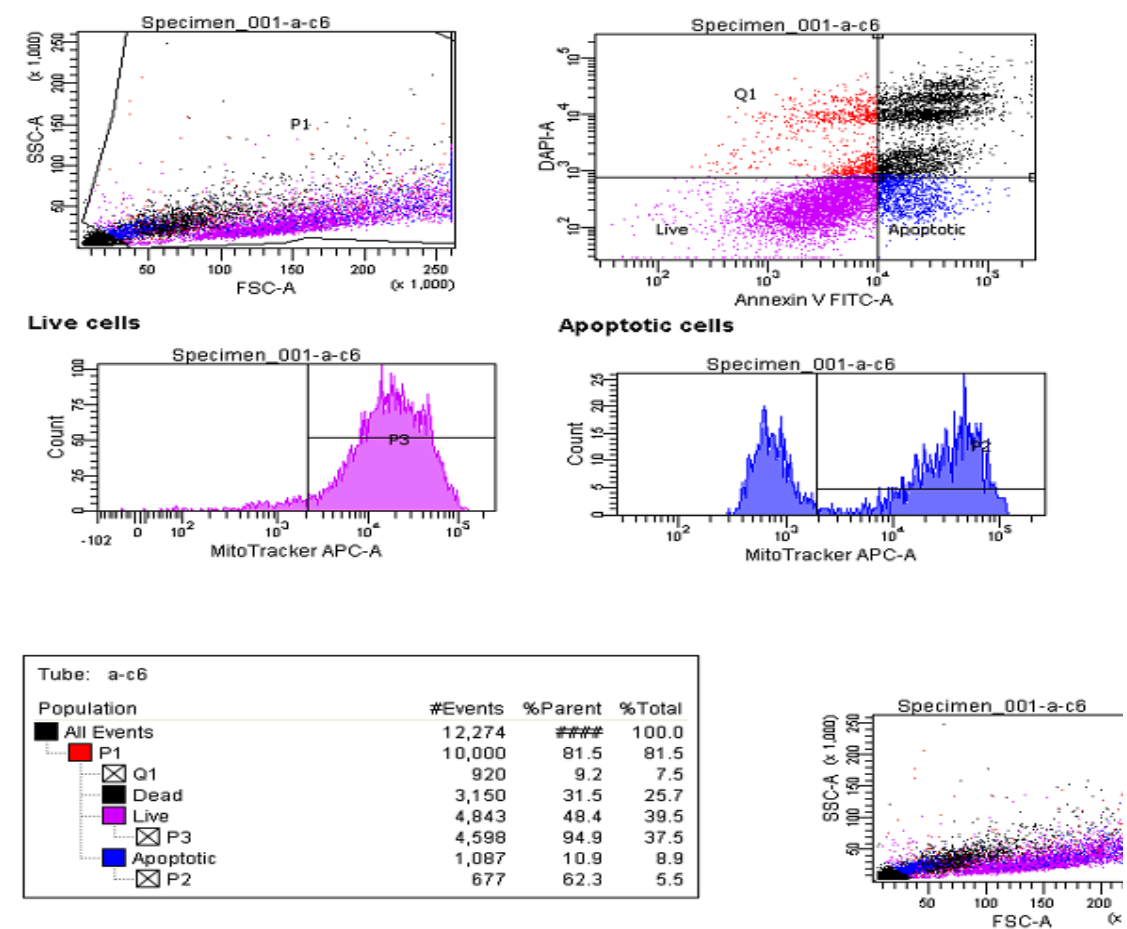


Supplementary Figure 5: FACS image for live and apoptotic differentiated PC12 cells after treatment with sera from a TS patient. The above flow cytometry images show the percentage of live and apoptotic PC12 cells differentiated into neurons after treatment with 1:100 sera from a TS patient. 15.5% of cells were found to undergo apoptosis after addition of sera antibodies. Only 5.2% cells were found to be alive out of which 19.4% had intact mitochondrial function.

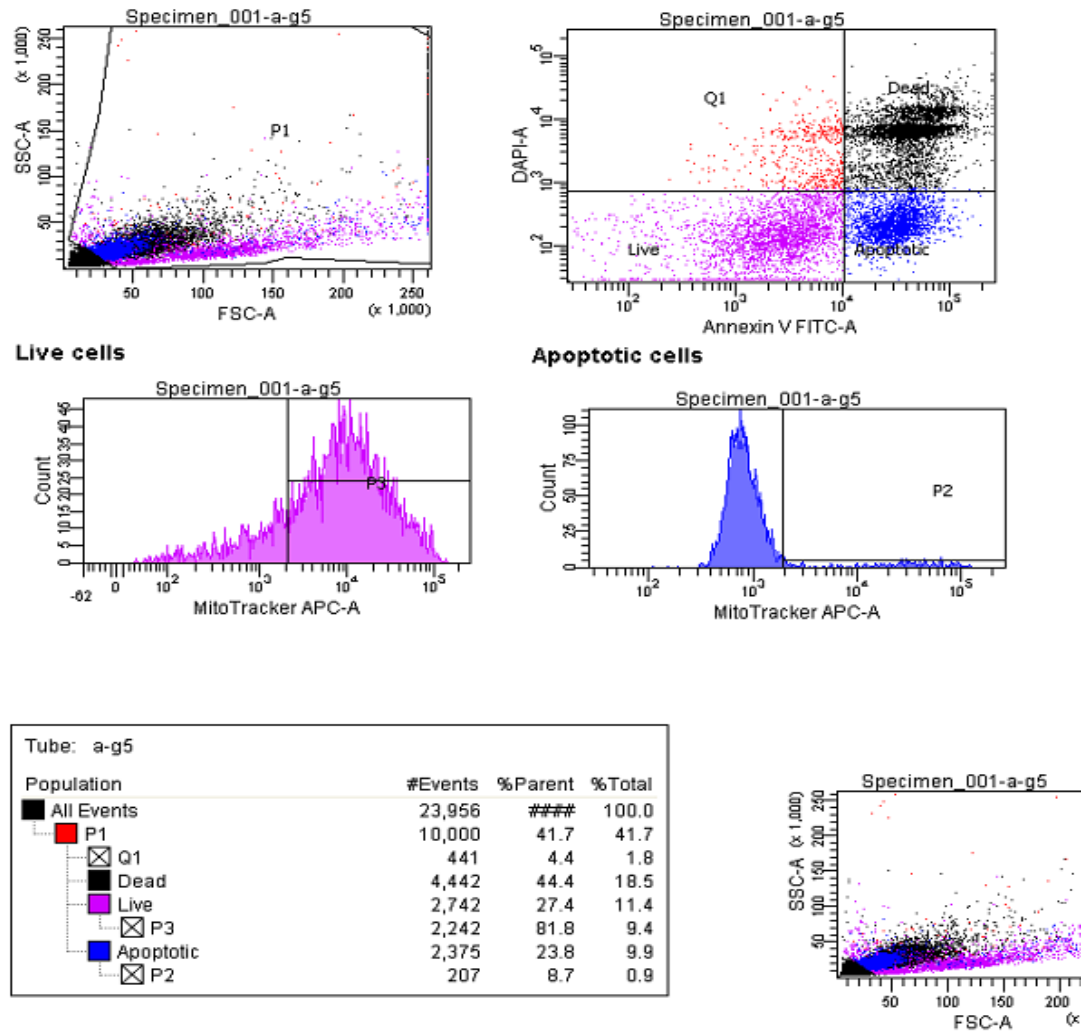


Supplementary Figure 6: FACS image for live and apoptotic differentiated PC12 cells after treatment with sera from a healthy control. The above flow cytometry images show the percentage of live and apoptotic PC12 cells differentiated into neurons after treatment with 1:100 sera from a healthy control. Only 6.1% of cells were found to undergo apoptosis after addition of control sera antibodies. 16.1% cells were found to be alive out of which 13% had intact mitochondrial function.

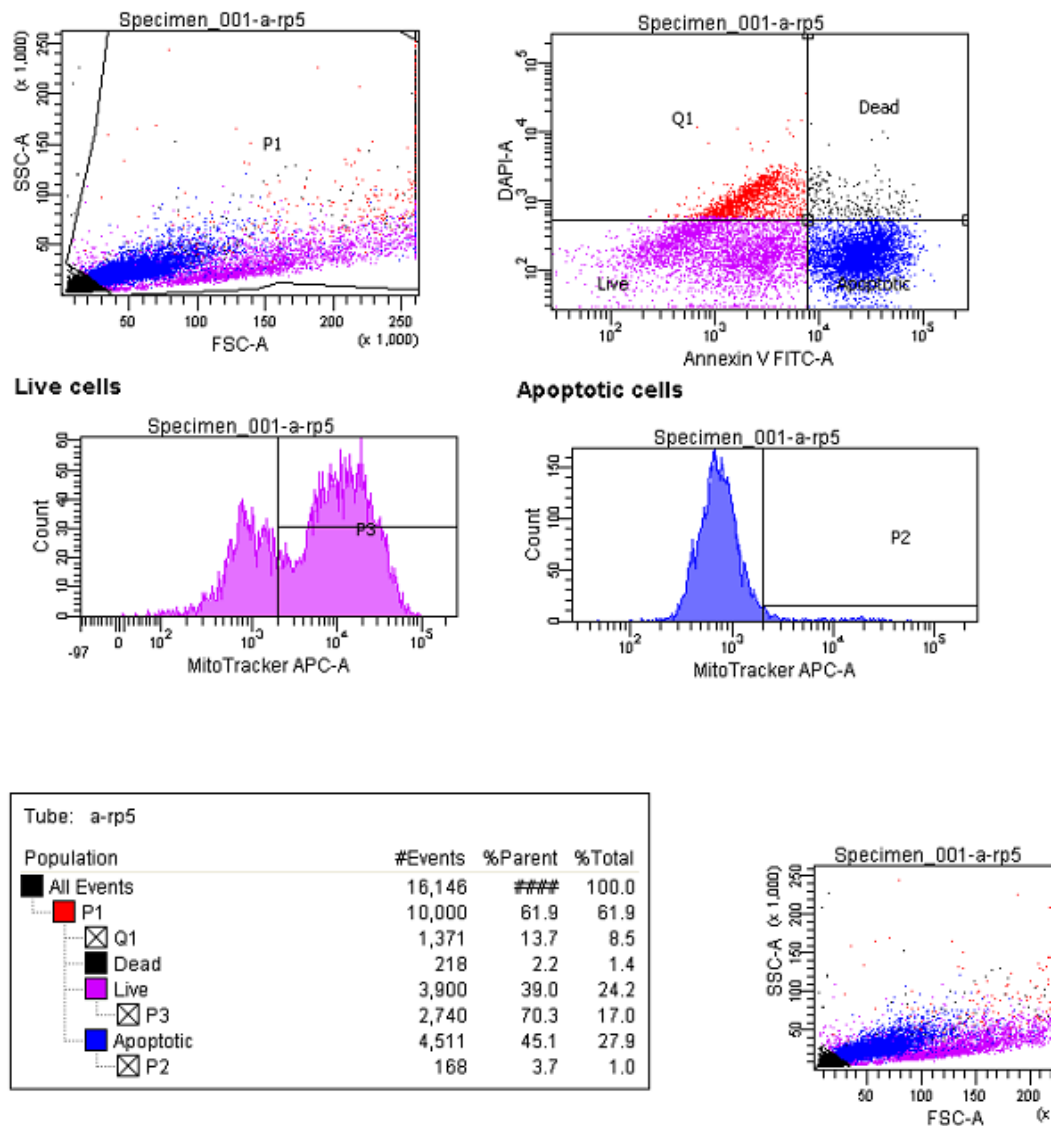
Undifferentiated PC12: ABH Animal Model



Supplementary Figure 7: FACS image for live and apoptotic undifferentiated PC12 cells after treatment with sera from ABH (CFA only) animal model. The above flow cytometry images show the percentage of live and apoptotic undifferentiated PC12 cells after treatment sera from ABH mouse immunized with CFA only. 10.9% of cells were found to undergo apoptosis after addition of sera antibodies. Only 48.4% cells were found to be alive out of which 94.9% had intact mitochondrial function.

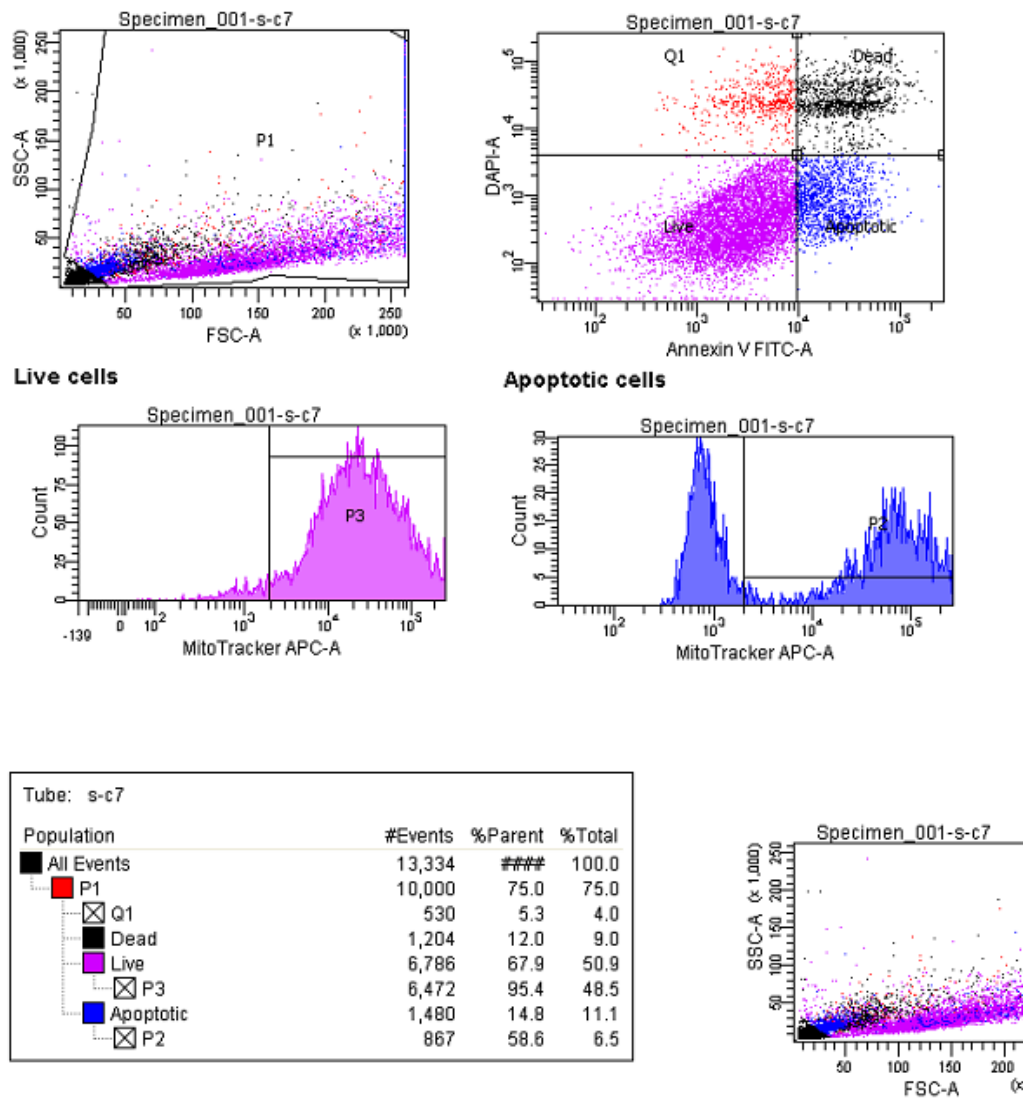


Supplementary Figure 8: FACS image for live and apoptotic undifferentiated PC12 cells after treatment with sera from ABH (GABHS) animal model. The above flow cytometry images show the percentage of live and apoptotic undifferentiated PC12 cells after treatment sera from ABH mouse immunized with GABHS protein fractions. 23.8% of cells were found to undergo apoptosis after addition of sera antibodies. Only 27.4% cells were found to be alive out of which 81.8% had intact mitochondrial function.

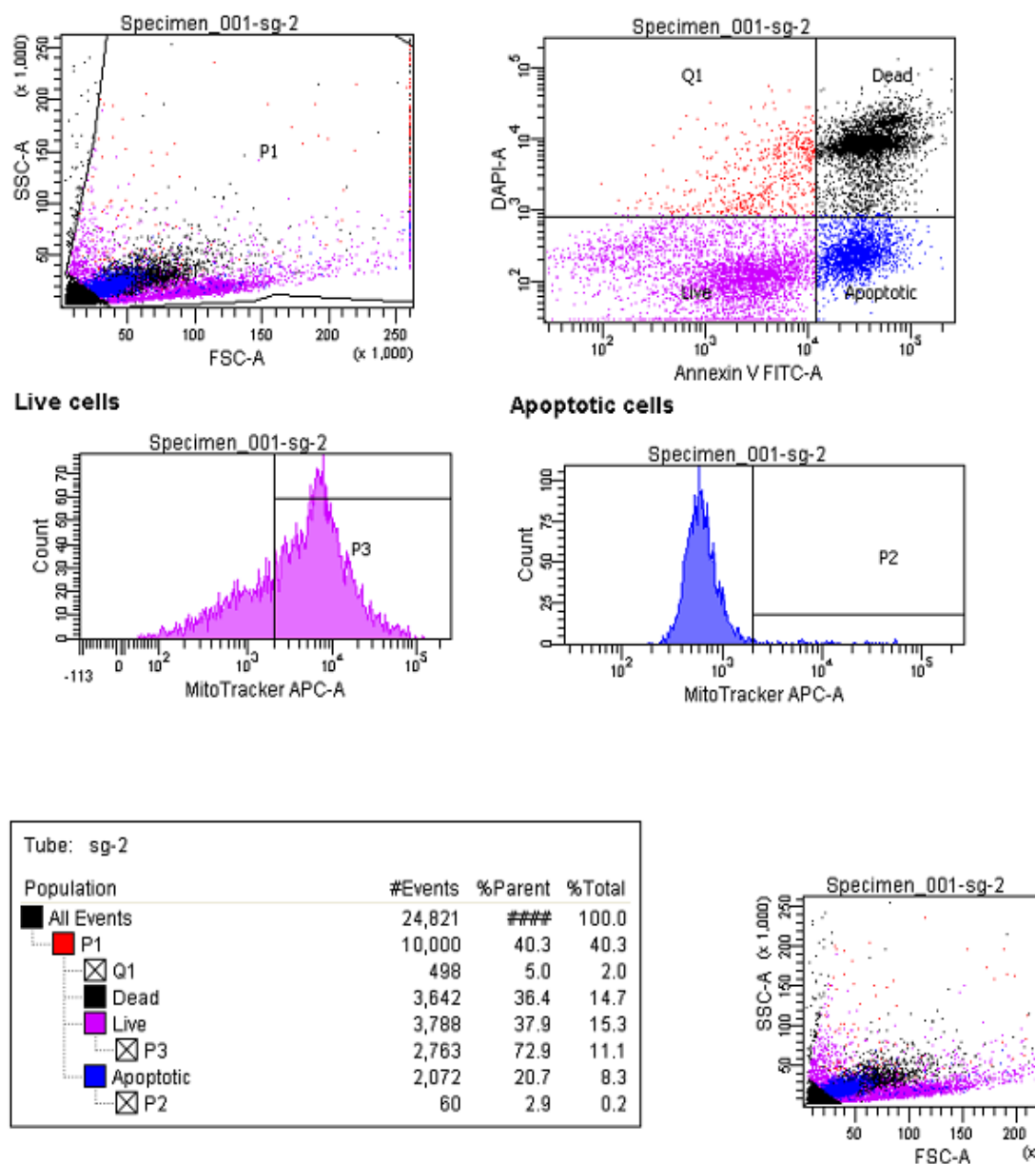


Supplementary Figure 9: FACS image for live and apoptotic undifferentiated PC12 cells after treatment with sera from ABH (RP) animal model. Supplementary figure 9: The above flow cytometry images show the percentage of live and apoptotic undifferentiated PC12 cells after treatment sera from ABH mouse immunized with recombinant proteins (aldC, enolase and PK). 45.1% of cells were found to undergo apoptosis after addition of sera antibodies. 39% cells were found to be alive out of which 70.3% had intact mitochondrial function.

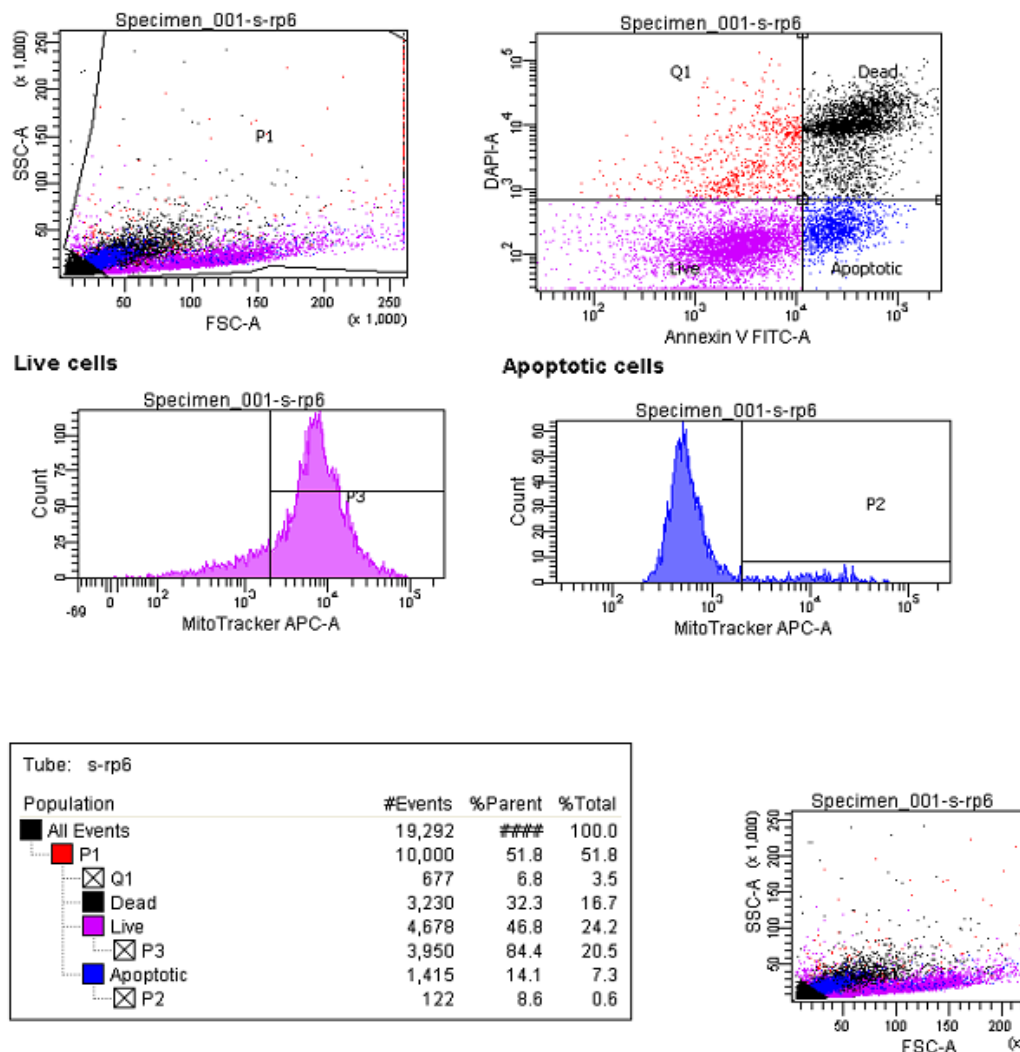
Undifferentiated PC12: SJL Samples



Supplementary Figure 10: FACS image for live and apoptotic undifferentiated PC12 cells after treatment with sera from SJL (CFA only) animal model. The above flow cytometry images show the percentage of live and apoptotic undifferentiated PC12 cells after treatment sera from SJL mouse immunized with CFA alone. 14.8% of cells were found to undergo apoptosis after addition of sera antibodies. 67.9% cells were found to be alive out of which 95.4% had intact mitochondrial function.

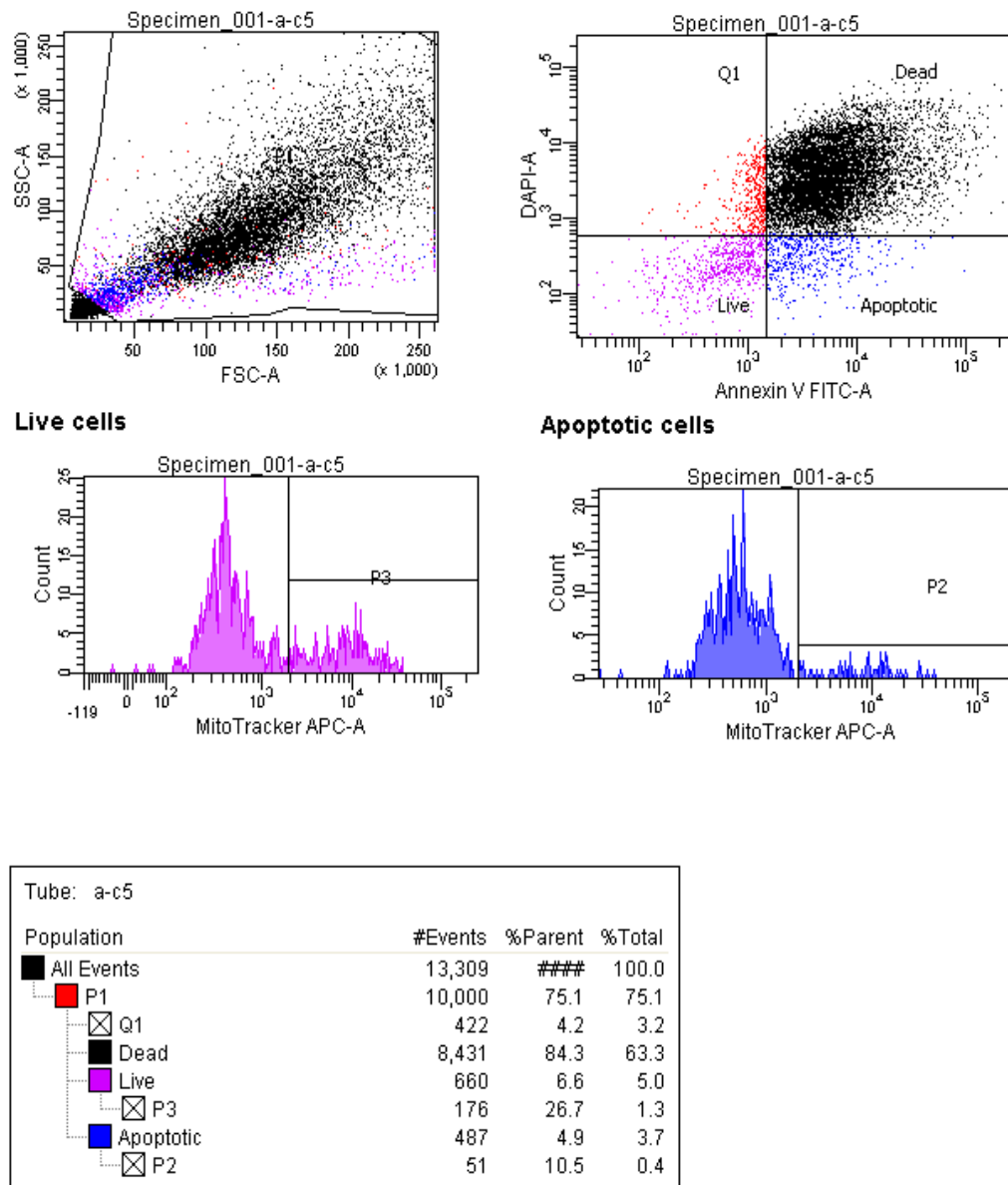


Supplementary Figure 11: FACS image for live and apoptotic undifferentiated PC12 cells after treatment with sera from SJL (GABHS) animal model. The above flow cytometry images show the percentage of live and apoptotic undifferentiated PC12 cells after treatment with sera from SJL mouse immunized with GABHS protein fractions. 20.7% of cells were found to undergo apoptosis after addition of sera antibodies. 37.9% cells were found to be alive out of which 72.9% had intact mitochondrial function.

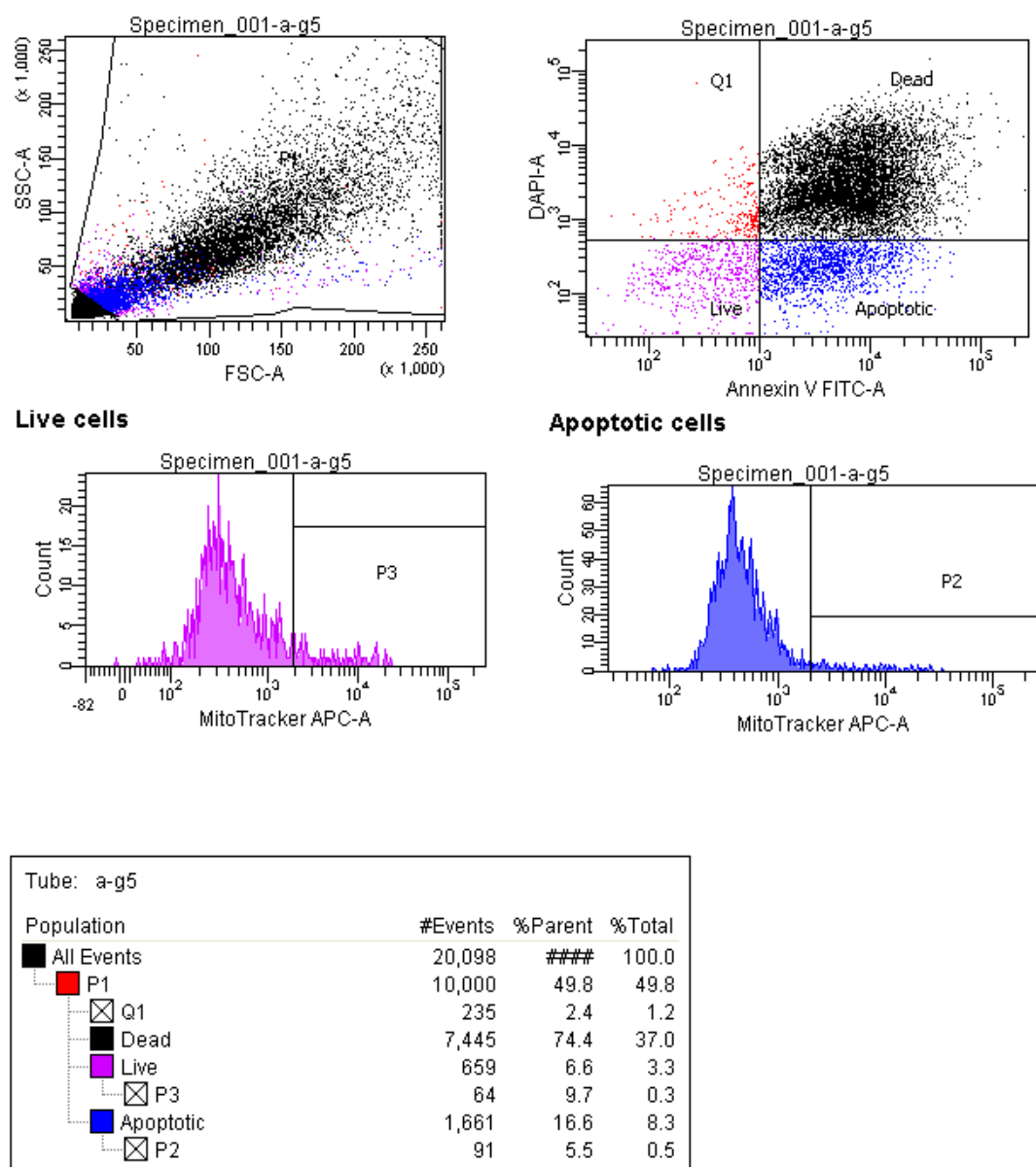


Supplementary Figure 12: FACS image for live and apoptotic undifferentiated PC12 cells after treatment with sera from SJL (RP) animal model. The above flow cytometry images show the percentage of live and apoptotic undifferentiated PC12 cells after treatment sera from SJL mouse immunized with recombinant proteins (ald C, enolase and PK). 14.1% of cells were found to undergo apoptosis after addition of sera antibodies. 46.8% cells were found to be alive out of which 84.8% had intact mitochondrial function.

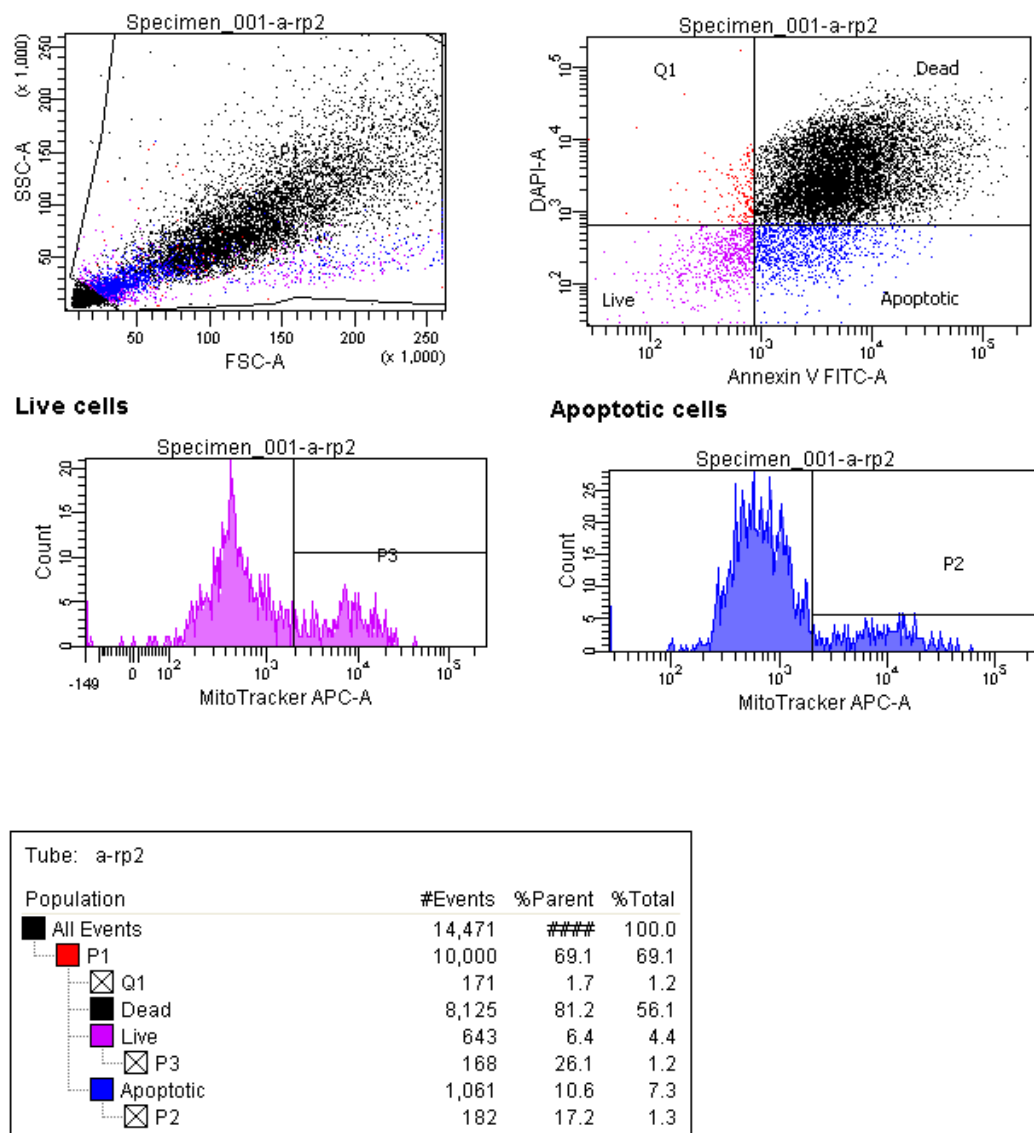
Differentiated PC12: ABH Samples



Supplementary Figure 13: FACS image for live and apoptotic differentiated PC12 cells after treatment with sera from ABH (CFA only) animal model. The above flow cytometry images show the percentage of live and apoptotic PC12 cells differentiated into neurons after treatment with sera from ABH mouse immunized with CFA alone. 4.9% of cells were found to undergo apoptosis after addition of sera antibodies. Only 6.6% cells were found to be alive out of which 26.7% had intact mitochondrial function.

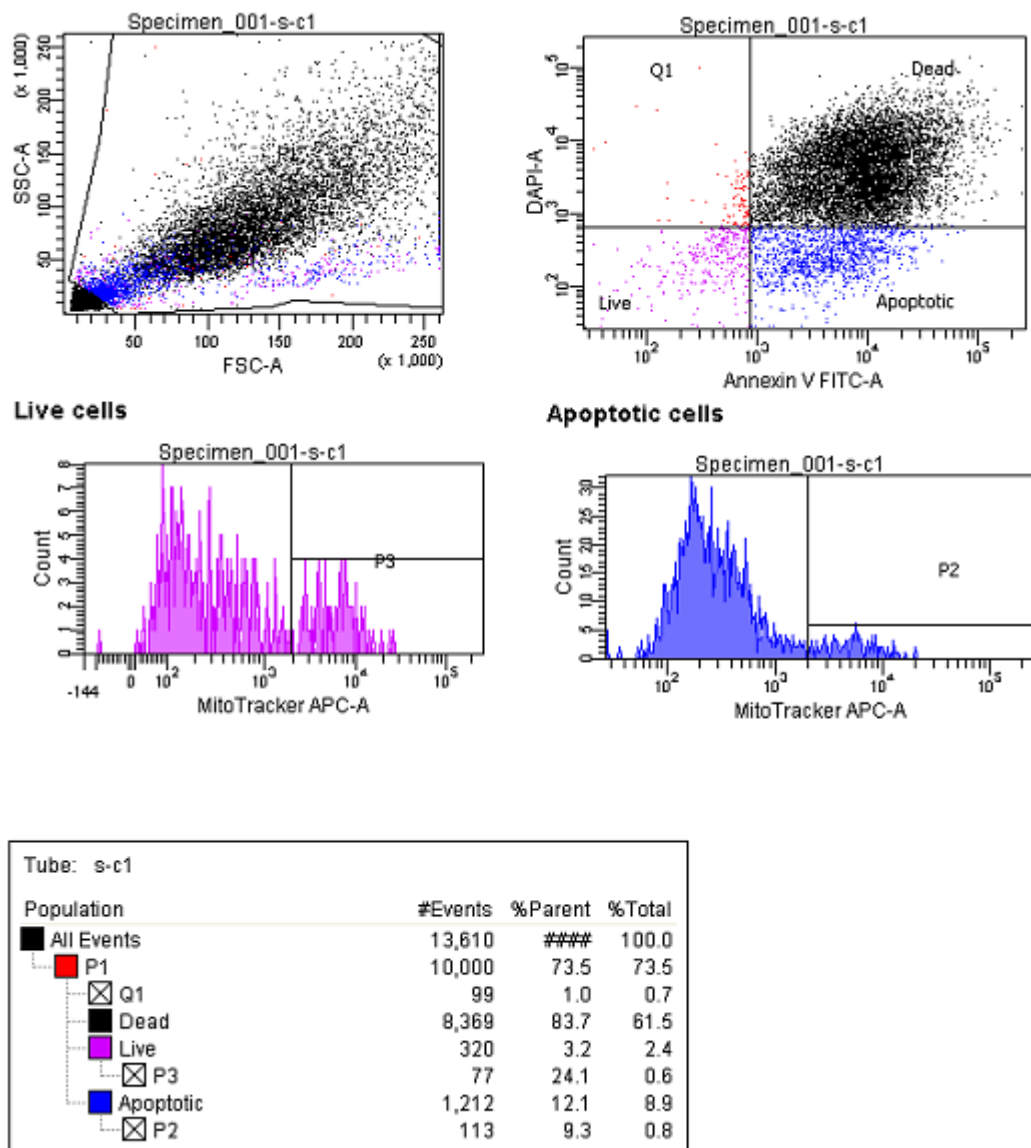


Supplementary Figure 14: FACS image for live and apoptotic differentiated PC12 cells after treatment with sera from ABH (GABHS) animal model. The above flow cytometry images show the percentage of live and apoptotic PC12 cells differentiated into neurons after treatment with sera from ABH mouse immunized with GABHS protein fractions. 16.6% of cells were found to undergo apoptosis after addition of sera antibodies. Only 6.6% cells were found to be alive out of which 9.7% had intact mitochondrial function.

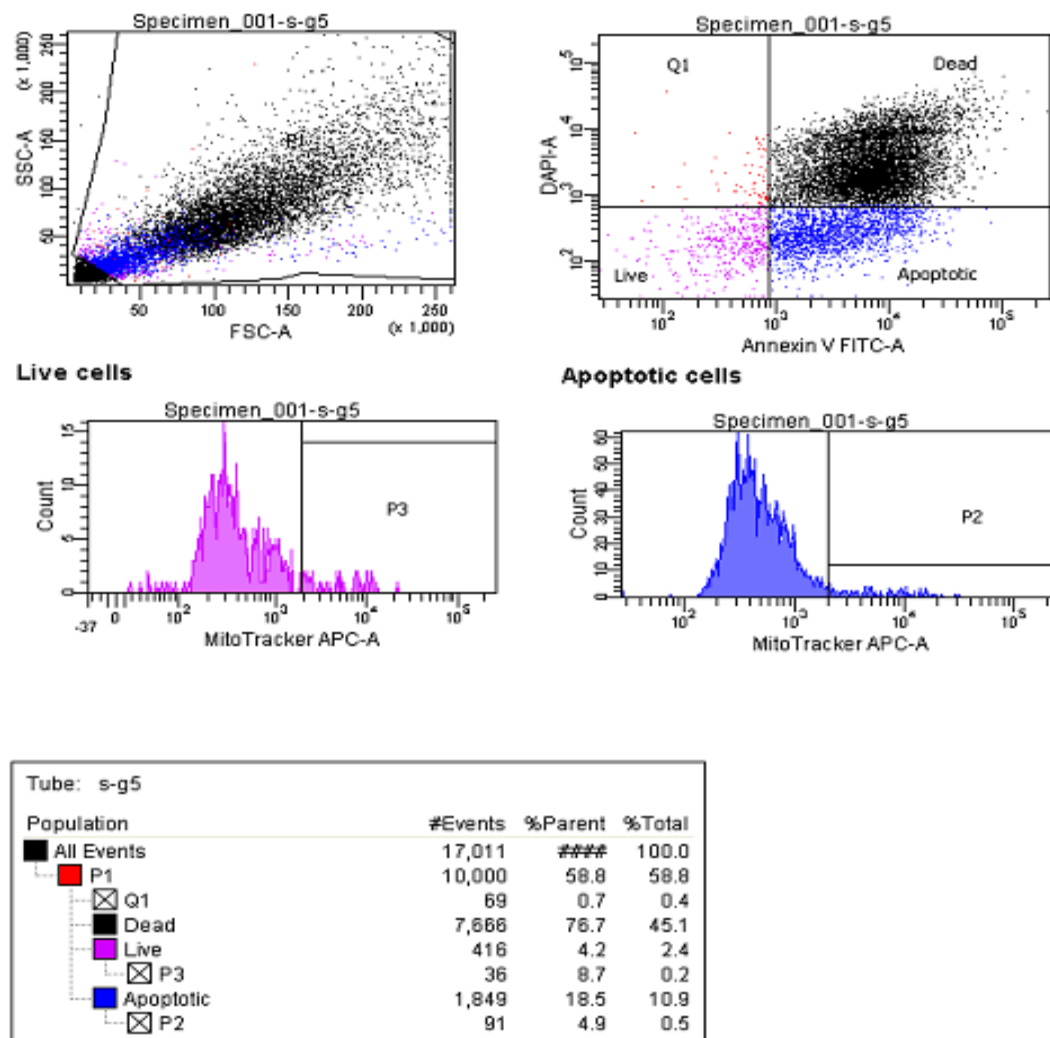


Supplementary Figure 15: FACS image for live and apoptotic differentiated PC12 cells after treatment with sera from ABH (RP) animal model. The above flow cytometry images show the percentage of live and apoptotic PC12 cells differentiated into neurons after treatment with sera from SJL mouse immunized with recombinant proteins (aldC, enolase and PK). 16% of cells were found to undergo apoptosis after addition of sera antibodies. Only 4.7% cells were found to be alive out of which 26.6% had intact mitochondrial function.

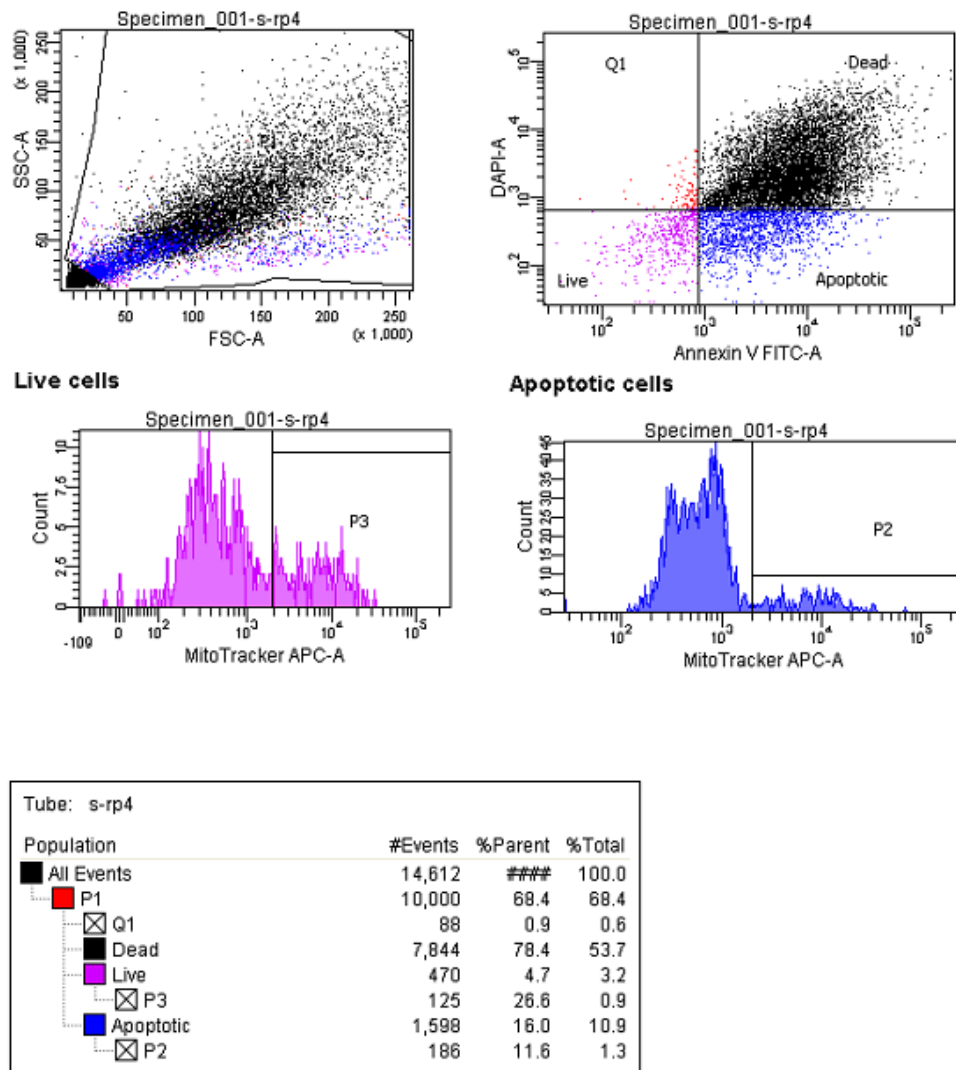
Differentiated PC12: SJL Samples



Supplementary Figure 16: FACS image for live and apoptotic differentiated PC12 cells after treatment with sera from SJL (CFA only) animal model. The above flow cytometry images show the percentage of live and apoptotic PC12 cells differentiated into neurons after treatment with sera from SJL mouse immunized with CFA alone. 12.1% of cells were found to undergo apoptosis after addition of sera antibodies. Only 3.2% cells were found to be alive out of which 24.1% had intact mitochondrial function.



Supplementary Figure 17: FACS image for live and apoptotic differentiated PC12 cells after treatment with sera from SJL (GABHS) animal model. The above flow cytometry images show the percentage of live and apoptotic PC12 cells differentiated into neurons after treatment with sera from SJL mouse immunized with GABHS protein fractions. 18.5% of cells were found to undergo apoptosis after addition of sera antibodies. Only 4.2% cells were found to be alive out of which 8.7% had intact mitochondrial function.



Supplementary Figure 18: FACS image for live and apoptotic differentiated PC12 cells after treatment with sera from SJL (RP) animal model. The above flow cytometry images show the percentage of live and apoptotic PC12 cells differentiated into neurons after treatment with sera from SJL mouse immunized with recombinant proteins (aldC, enolase and PK). 16% of cells were found to undergo apoptosis after addition of sera antibodies. Only 4.7% cells were found to be alive out of which 26.6% had intact mitochondrial function.